STUDIES ON DEVELOPMENT OF UNISTRAIN PROBIOTICS FOR ANTICANCER AND ANTIOXIDANT ACTIVITY

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DECLARATION

I, Mr. Abhinandan R Patil, hereby declare that the work presented in this thesis entitled **"Studies on development of unistrain probiotics for anticancer and antioxidant activity"** is entirely original and was carried out by me independently in the D. Y. Patil Education Society (Deemed to be University), Kolhapur under the supervision of Prof. (Dr.) S. H. Pawar, Emeritus scientist (CSIR); Distinguished Professor, D. Y. Patil Education Society (Deemed to be University), Kolhapur and Director, Center for Innovative and Applied Research, Anekant Education Society, Baramati and Prof. (Dr.) J. I. Disouza, Supervisor, D. Y. Patil, Education Society (Deemed to be University), Kolhapur and Principal, Tatysaheb Kore College of Pharmacy Warananagar, Kolhapur. I further declare that this study has not formed the basis for the award of any degree, diploma, fellowship or associate ship or similar title of any University or Institution. The extent of information derived from the existing literature has been indicated in the body of the thesis at appropriate places giving the references.

Place: Kolhapur

Date: 16/05/2019

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CERTIFICATE

This is to certify that the thesis entitled **"Studies on development of unistrain probiotics for anticancer and antioxidant activity"** submitted herewith for the degree of Doctor of Philosophy in **Pharmaceutical Sciences** to D. Y. Patil Education Society (Deemed to be University), Kolhapur by **Mr. Abhinandan Ravsaheb Patil** is absolutely based upon his own work under our supervision. Neither this thesis nor any part of it has been submitted elsewhere for any degree/diploma or any other academic award anywhere before.

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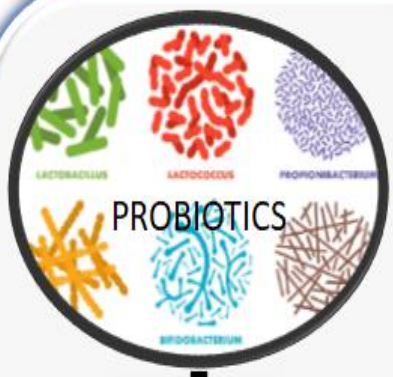
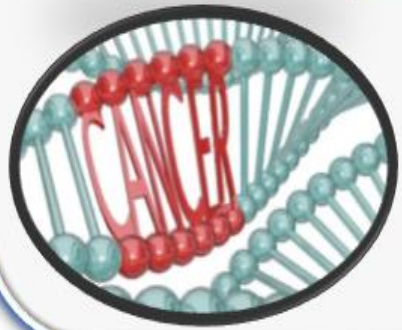
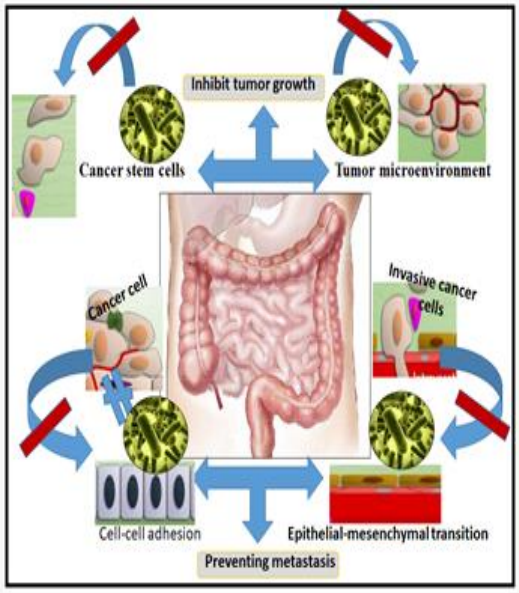
Chapter 1

Let food be thy your medicine- Hippocrates

Introduction







Cancer inhibition mechanisms by probiotics

1.1 Introduction

Cancer is considered as the disorder of the cell in which the natural death (apoptotic) pathway of the cell cycle is lost resulting in uncontrolled growth [1]. The World health organization (WHO) has declared cancer as the second leading cause of death universally with a mortality rate of 9.5 million people's in only 2018. The report further says that 1 in 6 deaths reported globally is due to cancer, in which 70% of deaths are from low- and middle-income countries. The main reason behind all these death are the 5 leading dietary and behavioral risks which include obesity, low intake of fruit and vegetable, sluggish physical activity, alcohol, and tobacco consumption [2]. Colorectal is the leading third most cancer in which nearly 8, 62, 000 deaths were reported in 2018 [3]. To cope up with this problem, few new synthetic medicines are discovered after several years of clinical trials and many are discarded at the last phase of trials due to host-drug incompatibility issues. The treatments of colorectal cancer are mostly given by the systematic route rather than the local action at the site of the disorder. This results in the systematic toxicity and adverse effects on vital organs. So there is a need of the drug that can be delivered by per-oral route with its local action, minimizing the systemic toxicity controlling morbidity and preventing the mortality [4].

The cost of these anticancer drugs and even certain antibiotics are beyond the reach of the common man and if made available are puzzled by the issue of innumerable side effects and suffering [5]. So, there is a need for such medicine which is from natural origin and deprived of its side effects [6]. With the advancement in the studies of nutraceutical foods and the knowledge of nano-pharmacology, the first of its kind new formulated functional food can be generated to heal colorectal cancer [7]. Most important thing is to deliver the functional foods by the per-oral routes directly into gastrointestinal tract like food bolus. They are promising due to the natural origin and can be made available at a low price especially in developing countries like India.

1.2 Cancer

Cancer is considered as the growth of cells, without its control of metabolism pathways resulting in the death of the host. Cancer affects each and every part and appears anywhere inside the human body. Normally, the cells grow and re-divide by mitosis by control on the G_1 phase. The loss of control on the G_o pathway results in the condition called malignancy [8].

The worn out or damaged cells are killed by programmed cell death/ apoptosis phenomenon, but the cancerous cells survive and form the extracellular outgrowth called benign tumors [9]. These tumors are the solid masses of host tissue where blood cancer is an exception called leukemias is liquid in nature [10]. Sometimes the tumorous cells have the abilities to spread and invade the surrounding tissue and organ and are called as malignancy [11]. These metastatic cells spread via blood and lymphatic system all over the body and are life-threatening. The normal cells matures in a distinct way in order to play a specific function inside the body, while cancerous cells grow in uncontrolled rate without any defined functionality as they ignore the cellular signals controlling the divisions [12].

The cancer cells develop their own new blood vessels called a neovascularization phenomenon for the purpose of the extra supply of the oxygen and nutrients as compared to normal cells. These cancerous cells invade the organ system, immune system and making prone the host to various infectious conditions. The current research proved that some of the cancer types are genetic in origin which may occur due to alternation in the genes controlling the functions of the particular cell. Different types of radiation such as X-rays, gamma rays and ultraviolet rays induces cancer [13]. Even different types of chemicals and environmental exposures such as smoke, pollutants and industrial effluents acts as a carcinogenic agent "cancer-causing mediators or mutagenic agent." The genes responsible for cancer are called oncogene or proto-oncogenes.

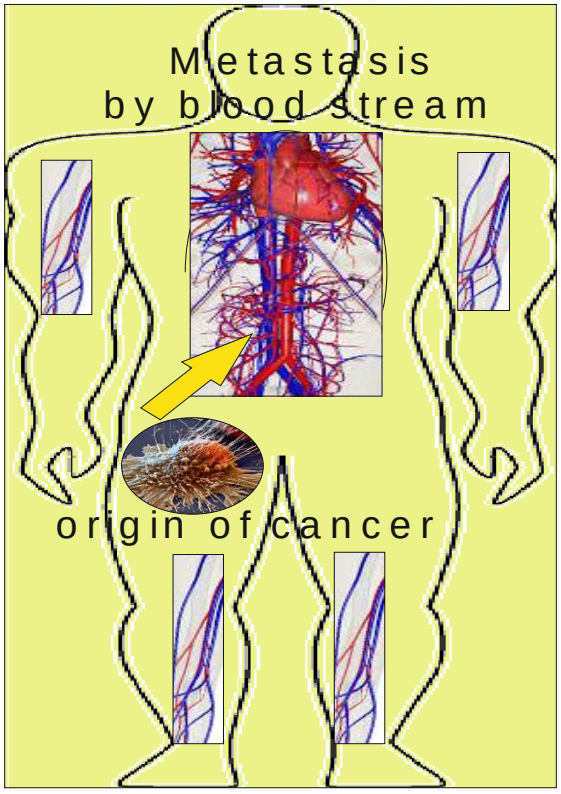


Fig. 1.1 The metastatic flow of cancer from the origin

The drastic and sudden changes in the morphological events of the cell cycle and wear-tear of body's tissues are not always cancer. The chronic and persistent irritation of the tissue results in the condition called hyperplasia [14]. The hyperplasia cells divides at a faster rate as compared to normal cells and look normal in morphology under microscopic staining. The more serious condition than hyperplasia is the dysplasia where the extra cells buildup is observed [15]. But microscopically these cells look abnormal with changes in tissue integrity and organizations. An exaggerative itchy or non-itchy mole fall under the criteria of dysplasia if not treated in time may develop into melanoma condition. The untreated dysplasia develops into the carcinoma [16].

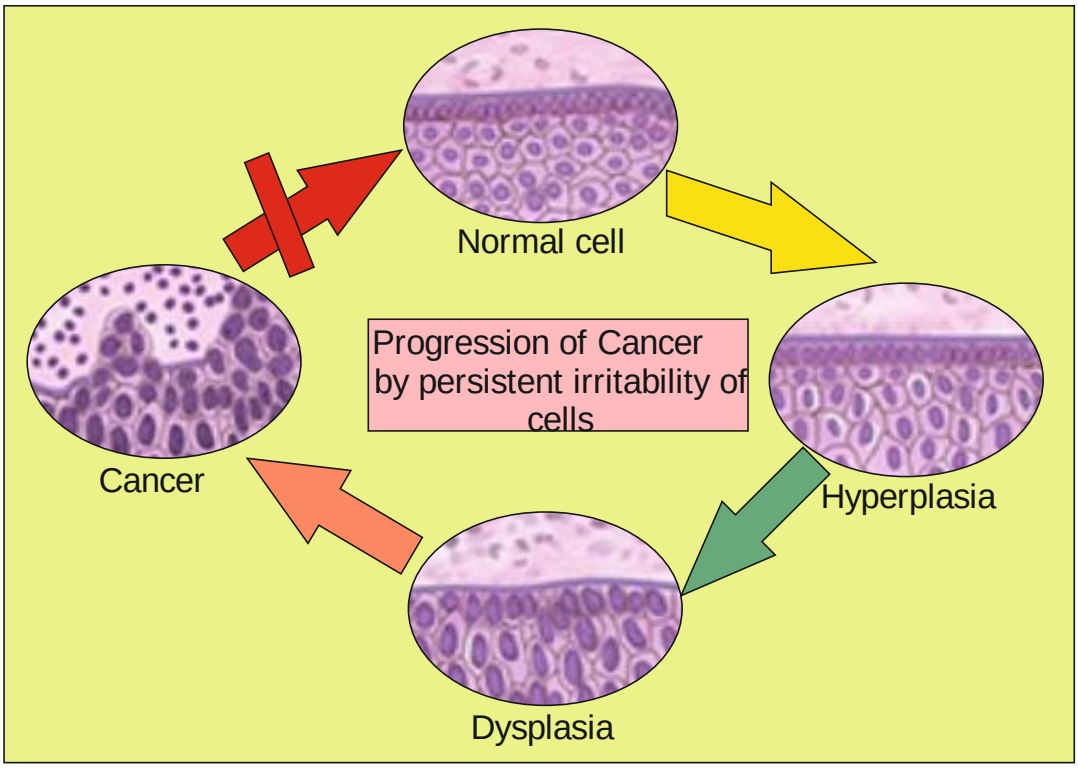


Fig. 1.2 The morphological progression of cancer in various stages

Thus, the persistent irritation of the cells develops the hyperplasia and then dysplasia. The probability that dysplasia develop into cancer is higher and it depends upon the physiology of the cells observed microscopically.

1.2.1 Types of Cancer

It's quite difficult to define the exact numbers of cancerous types the cell can develop [1]. It depends on the types of cells and their locations. The origin of the cancer may be from any organs, mostly it can be lung cancer or brain tumors. Mostly the type of cells affected by cancers such as glandular cells or squamous cells defines the intensity and its gravity [11]. Based on this system and for ease of understanding the cancer is divided as follows:

1.2.1.1 Carcinoma

The origin of this cancer is epithelial cells. These cells occur the inside and outside layer of the organ and organ system. Mostly the shape of these cells is column-like observed by microscopy [17]. Further, it's classified as adenocarcinoma,

in this type, there is the formation of mucus or fluid, observed inside the epithelial cells of the glandular tissues. This type of cancer is seen in the case of colon, breast and prostate glands. Similarly, the basal cell carcinoma is observed in lower strata of the epidermis layers of the skin surface. The carcinoma involving the squamous layer present above the epithelial cells includes the organs such as lungs, bladders, stomach, intestines, and kidneys called as epidermoid carcinomas [1, 18].

1.2.1.2 Sarcoma

Sarcoma form of cancer affects the soft tissues and organ system inside the body. Mostly it is observed in the case of fat, blood vessels, muscle, and tendons surrounding the joints. Osteosarcoma is a form of cancer observed in the case of bones, while the other type includes dermatofibrosarcoma-protuberans, malignant fibrous histiocytoma, Kaposi sarcoma, leiomyosarcoma and liposarcoma [19]

1.2.1.3 Leukemia

Cancers affecting the bone marrow or blood formation are called leukemias which never form the solid tumors. This cancer involves the presence of white cells in abnormal numbers with large crowd size in the blood vessels surrounding the normal blood cells. The increase in the number of these liquid tumors results in hypoxia and the sudden death of normal cells [10].

1.2.1.4 Lymphoma

The cancer of the lymphocyte system involving the T cells or B cells is called Lymphoma. These cells basically act as an immune-soldiers and fight against infectious conditions. In lymphoma, the level of lymphocytes increase abnormally in lymph nodes/ vessels and simultaneously in all other organs systems of the host. Basically, it is divided into two types as a) Hodgkin lymphoma is an abnormal increase in the lymphocytes count called as Reed-Sternberg cells and b) Non-Hodgkin lymphoma originates from B cells or T cells from the lymphocytes.

1.2.1.5 Multiple Myeloma/ Kahler disease

The origin of the cancer is from the plasma cells derived from the B cells of lymphocytes. The abnormal increase in the number and size of the plasma cells (myeloma cells) form the tumors of the bones [20].

a) Melanocytes cancer

Melanoma is a cancer of melanocytes. These cells are specialized in the formation of the melanin pigment. The melanin is an important component responsible for the formation of skin color. This cancer not only affects the skin surface but also eye morphology and its functioning called intraocular melanoma [11,20].

b) Brain and spinal cord tumors

These tumors are located in brains and spinal cord areas. These tumors are originated from astrocytes either as benign (mostly) or malignant.

1.2.1.6 Other types of tumors

The other types of cancer are germ cell tumors, neuroendocrine tumors and carcinoid tumors [18].

1.3 Colorectal cancer

The cancer of the colon or rectal areas depending upon the origin it is named as colorectal cancer. Mostly cancer develops from the polyps (growth in the inner lining of the colon/ rectum). The most common form of colorectal cancer observed is adenocarcinoma, while other forms are gastrointestinal stromal tumors, carcinoid tumors, colorectal lymphoma, etc. The congenital form of polyposis or non-polyposis is observed as an inheritance in origin [21].

The various types of colorectal cancers are as follows:

a) Adenocarcinoma

Adenocarcinomas originated from the internal lining of organs. "Adeno" name indicates as a gland. These tumors proliferate in the glandular region and affect the secretory functions. As like the breast and the lung cancer, these small adenomatous polyps if untreated spread as the malignant tumors [13,21].

b) Gastrointestinal Stromal Tumors (GIST)

These tumors originate in the digestive tract of the muscle tissues and are rarely observed at the colon site. Mostly these are benign (noncancerous) in nature, later may turn into malignant from which is termed as sarcomas form of cancer [22].

c) Lymphoma

A lymphoma form of cancer originates in a lymph node but in case of colorectal cancer, it initiates from the colon or rectum surface region. It includes the cutaneous lymphoma, non-Hodgkin's lymphoma, and Hodgkin's lymphoma [23].

d) Carcinoids

The hormone-producing specialized cells in the intestine are affected in case of Carcinoids cancer. These cancers are fatal in nature due to asymptomatic nature and are mostly treated by surgery.

e) Turcot syndrome

This condition is called an orphan disease and fall under the rare disorder category. Turcot syndrome is distinguished by colorectal polyposis, rectal cancer, and colonic tumors. The Mutations in MLH1, APC, and MSH2 genes results in the Turcot cancer [24].

f) Peutz-Jeghers syndrome (PJS)

It is classified as a syndrome due to its autosomal dominant disorder and further classified as [25]

I. **Melanocytic macules**

It is categorized by dark blue or brown moles on the body. The development of moles near or inside the mouth especially on the lips, surrounding the face and anus are the symptomatic conditions. These lesions spread till adulthood if ignored results into metastasis.

II. PJS is also present as multiple polyps in the colonic and rectal regions

III. PJS metastasis involves the risk of benign tumors of the testes and ovaries

IV. It further increases the risk of oesophageal and cancer

V. The mutation of the STK11 genes (tumor suppressor gene) is considered as the major reason for Peutz-Jeghers syndrome

g) Familial-colorectal cancer

The current research shows that about 15 % of colorectal cancer is hereditary in nature. This disorder is an autosomal dominant inheritance in nature explained by the family histories.

h) Juvenile polyposis coli

This is also an orphan or rare disease categorized as the autosomal dominant disorder. This disorder occurs due to the mutations in BMPR and SMAD/DPC genes. These cancer conditions are identified by the presence of the hamartomatous polyps in the colonic, small intestinal region and rectal parts. This disorder is identified by the various symptomatic conditions such as diarrhea, haemorrhage, enteropathy-associated with protein loss. Juvenile polyposis not only affects the gastrointestinal tract but also cause the pancreatic cancers initially at the beginning of metastasis.

1.3.1 Risk factors and etiology of colorectal cancer

Practically it is difficult to find the root causes of cancer. But there are many factors which may end with the occurrence of colorectal cancer termed as risk factors [21]. Encounter with one or more risk factors increases the probability of

colorectal cancer. Thus, the various factors which increase the possibility of cancer are:

a) Genetic risk

The current survey and research highlighted that person suffering from the Lynch syndrome, are prone to colonic cancer. In this disorder, the repair mechanism of the gene from cellular DNA fails, resulting in random gene mutations with the development of the endometrial and colorectal cancer. In these conditions, colon cancer is diagnosed mostly at the age of 30 years. Familial adenomatous polyposis is characterized by the colonic polyps and observed mostly in teenagers. Breast cancer and uterine cancer also increases the chances of colon cancer by the process of the metastasis [1,19].

b) Age and habits

The current research has shown that the person above 50 years of age is found prone to colorectal cancer. The wear and tear of the intestinal lumen along with aging increases the probability of gastrointestinal tract cancer. The old aged person with the history of the benign polyps is more prone to the adenocarcinoma type of colorectal cancer. In old age, the body fails to maintain vital organ integrity and is susceptible to many disease conditions. The habit of smoking increases the risk of colonic and rectal cancer. The carcinogens from a cigarette or from smoke when swallowed affect the digestive system by entering into the blood. This acts as a chemical agent which induces cancer in the gastrointestinal tract by eroding the intestinal lumen [26].

The incidence of colorectal cancer is found higher in obese people with wrong and unhealthy diet habits. Obesity develops due to the consumption of alcoholic beverages such as beer, a glass of wine, etc. The consumption of alcohol is having a close resemblance with the obesity thus acts as a triggering factor for this disorder. The inclusion of the red meat diet such as pork, beef, and lamb increases the risk of colorectal cancer compared to pure vegetarian people. This type of diet loads the excessive fat content in the intestinal lumen with the elevation of low-

density lipoprotein serum cholesterol level. Many researchers have shown that cooked meat at a high temperature such as "kabab" produces the carcinogenic substances during the processing of these food articles [21,26].

c) Chronic disorders

Type 2 diabetes share the common risk factors as that of colorectal cancer. The factors such as obesity, high blood pressure affecting the Type 2 diabetes increases the incidence of colorectal cancer. Similarly, the person suffering from the inflammatory bowel disease (IBD) such as Ulcerative colitis and Crohn's disease if not treated suffer from colorectal cancer. These above disorders changes the physiology of the intestinal epithelial lumen into hyperplasia and dysplasia conditions resulting in neovascularization. Even polyps development in IBD creates the worst prognosis and detection of the carcinoma [21,27].

1.3.2 Symptoms

The symptoms observed in a patient depends upon his/ her pathophysiological condition and prognosis. The symptoms also drastically vary from age, gender, diet, and habitat of the individuals. In general, the common symptoms shared by the patients include [13,26]

a) Change in the bowel movements due to the incidence of diarrhea, constipation, or stool narrowing
b) Anal bleeding with the darkening of the stools
c) Stomach pain with the cramps or gnawing
d) Loss of the appetite
e) Nausea and vomiting
f) Weight loss
g) Severe weakness and fatigue
h) Breakdown of jaundice resulting in yellowing of the body and skin
i) The worsening of the bowel habitat by a secondary infection caused by the unidentified hemorrhoids may be confused as chronic piles or fissure like conditions.

1.3.3 Treatments

The line of treatment depends upon the following condition such as [28]:

I. The patient's pathophysiology and other medical condition
II. Patients current health status
III. Allergy or hypersensitivity against different medicines
IV. Nutritional status, financial and social support to the patient

The gold standard line of treatment for the colorectal cancer are:

a) Surgery

Surgery includes the removal of the tissue of the tumor as a part of surgical resection. Basically, during operation, a part of the healthy colon or rectum surrounding the lymph nodes are removed. This surgical procedure is carried out by colorectal surgeons (proctologists). The supportive surgical options as a mode of treatments include [19,29]

I. Laparoscopic surgery

II. Colostomy for rectal cancer

III. Cryoablation or radiofrequency ablation (RFA)

Side effects of surgery includes the postoperative severe pains and tenderness at the site of surgery. The loss of intestinal rhythm results in constipation or long-term diarrhea. The colostomy patient has persistent irritation surrounding the stoma.

b) Radiation therapy

Radiation therapy is used to destroy the carcinogenic cells which include the use of x-rays of high energy. The various techniques employed in these therapies includes the use of external-beam radiation to shrink the developed tumors. Similarly, stereotactic radiation therapy is used to treat the tumors that have spread to different vital organs such as the liver, etc. This equipment dumps large but

precise radiations to a desired specific small area of tumors. This therapy prevents the surgical removal of the damaged organs and prevents malignancy [21,30].

Side effects of radiation therapy include fatigueness, mild skin reactions, stomach upsets and irregular bowel movements. The adverse side effects include the bloody stools with loss of control on the anal sphincters. Sometimes it resulted in sexual problems including infertility after pelvic region radiation therapy.

c) Chemotherapy

The long-term use of the drugs and chemicals for destroying the cancer cells are called chemotherapy [28]. This therapy is carried out by the medical expert doctor called an oncologist. The chemotherapy drugs are introduced into the systemic route by various methods. It includes by the intravenous (IV) or enteral route. The chemotherapy dose regimen is given in cycles for a particular period of time to destroy the cancer cells. The patients are given a single drug or combinations at the same time or different time intervals. Chemotherapy is preferred after surgery to prevent the widespread of the cancer cells. In the case of colorectal cancer, the chemotherapy is given before the surgery to shrink the size of the rectal tumor and to minimize the complication chances during the surgery [28,31].

I. Chemotherapy drugs approved for colorectal cancer

The U.S. Food and Drug Administration (FDA) globally has approved many drugs to treat colorectal cancer [28,31]. Few of the synthetic drugs used for the treatment along with its brand names are:

i. Capecitabine (Xeloda)
ii. Tipiracil (TAS-101/2), etc.

The combination therapy includes the use of:

i. 5-FU with leucovorin
ii. Capecitabine, along with an oral form of 5-FU
iii. Capecitabine with irinotecan

iv. Capecitabine with oxaliplatin

v. Combination of cetuximab, bevacizumab, or panitumumab.

II. Side effects of chemotherapy

The use of synthetic origin chemotherapy drugs comes with the side effects which includes vomiting, diarrhea, nausea, mouth sores, and neuropathy. In addition, there is increased chances of the infection due to the downfall of the immune system during this therapy. Numbness of body especially hands, with the significant loss of hair is the uncommon side effect observed during this therapy.

d) Targeted therapy

Targeted therapy is the most favored line of treatment concentrating cancer's specific proteins or the cancerous tissue environment. This treatment targets mostly the cancer cells as compared to normal cells, prevents the growth and spread of cancerous cells.

1.4 Alternative curative/ prophylactic methods against colorectal cancer

Many holistic systems use the combinations of medicinal plants along with the fruits and vegetables containing the phytochemicals such as phenolic compounds, vitamins (vitamin A, C, and E) and fibers to prevent and treat cancer [32]. These component acts as functional food which along with nutrients provide the health benefits called nutraceuticals. Many green leafy vegetables and season fruits act as a functional food and provide health benefits. The many exotic nature of the medicinal plants is used by the tribal community to heal many kinds of bodily disorders since ancient times [7].

1.4.1 Polyphenols and phytochemicals

Polyphenols are the group which contains different medicinal component such as simple phenols, acetophenones, stilbenes, hydroxycinnamic acids, coumarins, lignans, phenolic acids, flavonoids, and tannins. These components are found effective in blocking the telomerase in cancer cell lines proved by many *in-*

vitro and *in-situ* studies [33]. The detailed illustration of the polyphenols and their phytochemicals are described below [34]

Table. 1.1 Class of polyphenols with the types of phytochemicals

Sr. No.	Class of polyphenols	Phytochemicals
1	Acides phenols	Gallic acid
		Capsaicin
2	Phenylethanoids	Tyrosol
3	Acide hydroxycinnamique	Curcumine
4	Phenylpropenes	Eugenol
5	Phenylpropanes	6-Gingerol
6	Coumarines	Umbelliferone
7	Xanthones	Ziganein
8	Stilbenes	Resveratrol
9	Flavonoides	Quercetin
10	Lignanes	Pinoresinol
11	Bioflavonoids	Amentoflavone
12	Isoflavonoids	Genistein
13	Phenylacetiques	Caffeic acid

Along with the cell lines and animal model studies, the anticancer activity of the phytochemicals is identified by the biomarkers studies [32]. Apart from the phytochemicals, the other nutraceuticals agents include the fats and oil, dietary

fibers, plant secondary metabolites, medicinal herbs and few of the microorganisms [35 36].

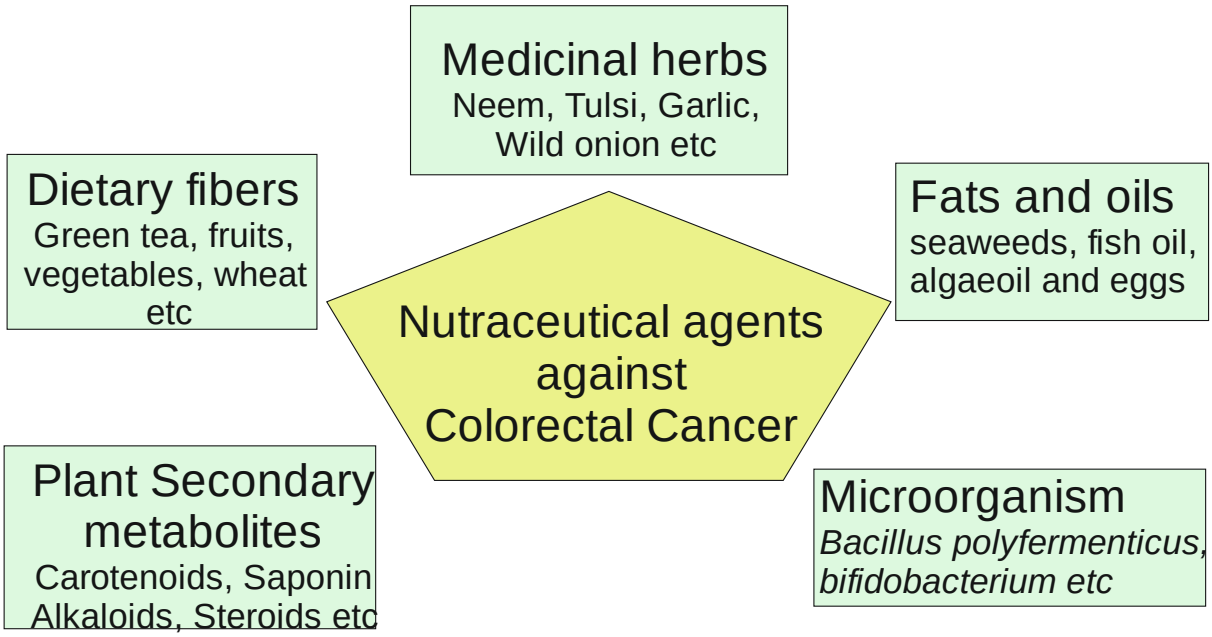


Fig. 1.3 Nutraceutical agents used against the colorectal cancer

Table. 1.2 Plant-source anticancer effects on the colon cancer cell lines

Sr. no.	Plant source	Type of cell line
1	Leaves of the olive tree	HT 29 colon cancer
2	Milk-based products	HT 29 colon cancer
3	Fruit juice of apple	SW 620 colon cancer
4	Allium fistulosum	CT 26 colon cancer
5	Garlic extract	Caco 2 colon cancer
6	Centaure-a ainetensis	HT 29 colon cancer
7	Oats extract	HCT 116 colon cancer
8	Centella asiatica extract	Caco 2 colon cancer

9	Mushroom extract	HCT 116 colon cancer
10	Grapes wines juice	Caco 2 colon cancer
11	Cichorium endlvia. extract	Caco 2 colon cancer
12	Allium sativum extract	HT 29 colon cancer

Many anticancer *in-vitro* cell lines studies were shown by the use of different fruits, vegetables, and plant extract.

The antioxidant mechanism is the mode of action observed in the case of most of the nutraceutical agents against colorectal cancer [7,36].

Table. 1.3 Antioxidant mechanism of nutraceutical agents at the molecular level

Nutraceuticals agents	Antioxidant mechanism of action at the molecular level
α-Tocopherol	Antiproliferation of tumor cells by the oxidative phosphorylation
Silibinin	Inhibit the cancer cell growth at the G_0 phase
Fenugreek	Activates cytokines
Curcumin (haldi)	Stimulates the MAPk genes showing anticancer activity
Iron-containing fruits and vegetables	Acts on transferrin receptor (TfR1)
Grape Seeds	Control the overexpression of the oncogene
Zerumbone	Upregulate the DR4 genes

Allicin	Inhibit the overexpression TNF-α family gene
Rhizocephalan	Activates the caspase-3 gene
Fucoxanthin	Induce apoptosis
Protein and peptides (milk)	Hyperproliferation of epithelium
Garcinol	Inhibition of tyrosine phosphorylation
Xanthohumol	Activates the caspase-3, -8, -9
Boswellic acid	Downregulation of cyclin D1
β-Escin	Arrest the cell cycle at the G1 and S phase
Quercetin	Downregulation of cyclin D1

Most of the nutraceuticals or functional food are useful and are free from side effects which are not seen in case of chemotherapy based synthetic drugs.

1.4.2 Probiotics as a functional food

The probiotics are considered as the class of microorganism that are found as residential microflora of the gastrointestinal tract assisting the digestive and enzymatic function of the host [37]. These friendly microorganisms are resident of the gastrointestinal tract of host since birth and can be introduced by means of functional food from plant or animal sources. The new-born babies not only receive IgA from the milk of the mother but also get inoculation cultures of the Lactobacillus [38]. In the gut of babies, the journey of Lactobacillus is affected by the dysbiosis phenomenon between the pathogenic strains and probiotics microbial flora. The

current research has shown that the concentration of these microbes falling in between the therapeutical window acts as prophylactic and curative agents against different diseases of the host bowel [39].

1.4.3 Antioxidant nature of the probiotics

Recent studies in relation to probiotics have proven that these microbes as living non-pathogenic microbiota after the administration in appropriate doses acts as antioxidant mediators preventing and curing many diseases and disorders. This microbiota shows different pharmacological and physiological activity that plays a vital role in the human immune system [40]. Many of the chemicals released by the Lactobacillus strains acts like SOD (superoxide dismutase) which role-plays as antioxidant mediators. These microbes also chelate various metal ions during the digestion process. *Lactobacillus casei* proved to chelate Fe2+ or Cu2+, due to its antioxidant nature [41].

Many of the signaling mechanism assisted by the probiotics microbes involve multistep complex pathways. In this Nrf2-Keap1 deals with the transcriptional responses by eukaryotic cells by exogenous sensitization [42]. NFκB deals with the transcriptional responses by eukaryotic cells due to excessive oxidative stress loading in the host cell. The anabolic processes involving the cell growth cycle along with its division is governed by the MAPK. Its subtypes whereas p38-MAPK and JNKs are linked with the diverse stresses encountered by the cell affected by osmotic shock and irradiations. The following table illustrates the mechanism of signaling pathways assisted by the probiotics microbes on the different host cell [40,43].

Table. 1.4 Signaling pathway regulated by the probiotics in relation to antioxidant activities

Probiotics	Host cells	Signaling pathways
Clostridium butyricum	Rats	Nrf2-Keap1
L. rhamnosus GG	Cell line Caco2	MAPK
Bacillus amyloliquefaciens	Cell line 'IPEC-1 cell line'	Nrf2-Keap1
Lactobacillus sp. SC4	Mice	Nrf2-Keap1
Lactobacillus sp. CM	YAMC	MAPK
Lactobacillus sp. FC255	Cell line Mice	Nrf2-Keap1

The probiotics show antioxidant activities by producing various metabolites involving folate and glutathione (GSH). The folate content from the vitamins regulate the vital metabolism signaling pathways. This folate act as linkage to complete the process of DNA replication, methylation and maintaining the wear and tear of the host cells. This folate act as crucial metabolic part in the antioxidant activity. Evidence-based studies have shown that Bifidobacteria induces the folate formation in animal models and human trails. Further investigation showed that the *Lactobacillus fermentum* exhibit the GSH system regulation [40,44]. Thus, it is concluded that the levels of antioxidant metabolites enhanced by the probiotics treatments acts as natural antioxidant agents. The deficiency of the vitamin B12 and folate induces excessive oxidative stress, precipitating type 2 diabetes in the adult. The researcher showed that consumption of the yogurt, rich in Lactobacillus species improves the level of the vitamin B12 along with the plasma folate relieving the oxidative damages in a diabetes patients [40,45]. The detailed illustration of the antioxidant mechanism exhibited by the probiotics are as follows:

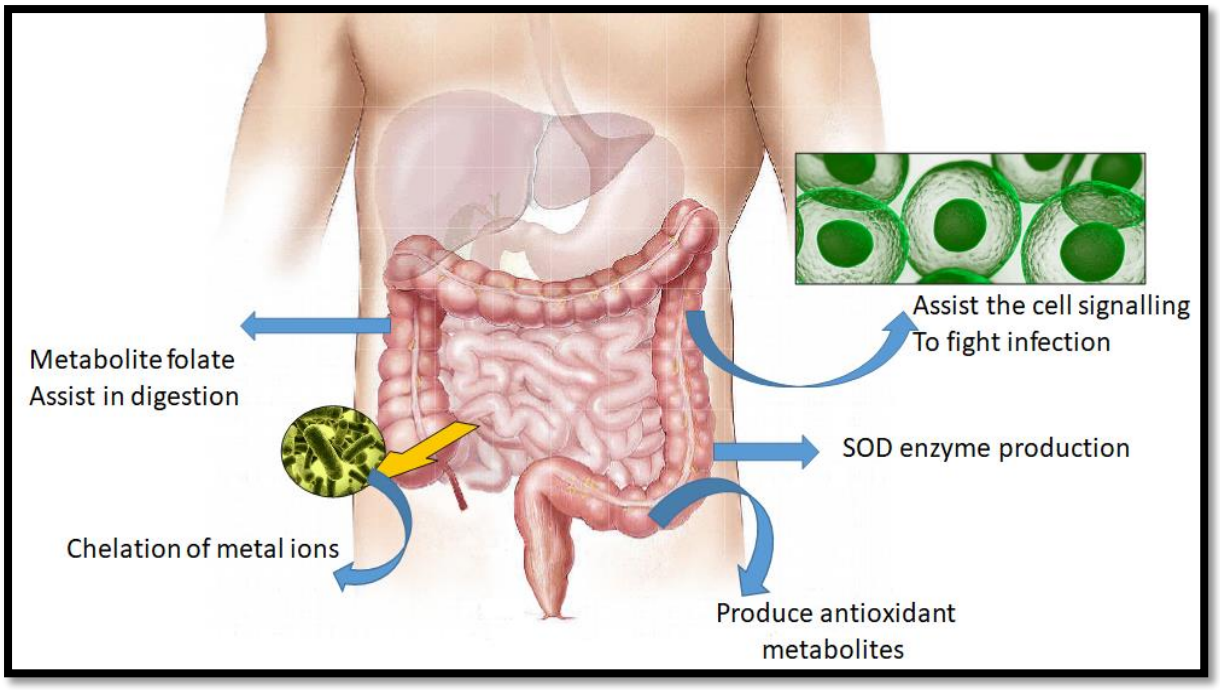


Fig. 1.4 Antioxidant activities exhibited by the probiotics involving a various regulatory mechanisms

1.4.4 Anticancer nature of the probiotics

Lactic acid bacteria also are known as Lactobacillus a genus from the probiotics group is found useful in healing various common disorders and even conditions like cancer [46]. These cancer healing abilities exhibited by the probiotics includes various mechanisms such as suppression of cancer-causing mediators [47]. This is regulated by preventing the infestation of altering carcinogen metabolites by DNA protections. In addition, probiotics participate in the proper execution of the cell apoptosis preventing the cancer invasion by metastasis and proper maintenance of the cancer stem cell by killing cancer inducing signaling [48]. The various mechanisms shown by the probiotics against cancer cells are as follows [48–50]:

a) Protecting host by cell-cell adhesion

Normal physiology of the host cells consists of the tight junction of the endothelial system surrounded by the epithelial showing the cellular integrity. During the metastasis, these structures are weakened by the damaging of the scaffold of protein composed of occludin and zona occludens-1 [48]. Sometimes the host cell membrane degrading agents such as matrix metalloproteinases assist the cancer cells to invade the cellular structure by damaging its integrity [51]. In addition, the use of the probiotics before surgery in a patient is found to enhance the liver barrier preventing the metastases [40,52,53]. Lactobacillus is found effective against cancer due to its cell adhesion mechanisms. *L. rhamnosus* was found to decrease the overexpression of matrix metalloproteinase-2 in $Caco_2$ cell line maintaining the cell-cell adhesion [3,48,54].

Table. 1.5 Cell-cell adhesion mechanism exhibited by the probiotics

Sr.no	Probiotics	Cell line/host cell	Cell-cell adhesion mechanism [3,48,49, 52, 54, 55].
1.	*L. acidophilus*	Human monocytes	Up-regulation of metalloproteinases (TIMP)-1 as a tissue inhibitor
2.	*L. rhamnosus* GG	Human monocytes	Up-regulation of metalloproteinases (TIMP)-9 as a tissue inhibitor
3.	*L. rhamnosus* GG	MDA-MB-231 cells	Down-regulation of *GLUT1*
4.	*L. acidophilus*	HT 29 cell line	Increase in *ICAM5 expression*
5.	*Lactobacillus species* (NCK2025)	HT 29 cell line	Up-regulation of TIMP-2

6.	Kefir	4T1 cell line	Down-regulation of plasminogen activator urokinase
7.	Kefir	BALB/c mice	Enhancement of helper T and cytotoxic T cells
8.	Kefir	Caco 2 cell line	Up-regulation in Bax
9.	Kefir	HT 29 cell line	Expression of p53 independent of p21 induction
10.	*L. plantarum*	Caco 2 cell line	Translocation of ZO-1 in cell junction
11.	*L. rhamnosus* GG	Caco 2 cell line	Translocation of in cell junction
12.	*L. rhamnosus* GG	Caco 2 cell line	Up-regulate of Claudin-1 in cell junction

b) Protecting the host by inhibiting the epithelial-mesenchymal transition (EMT)

It is the part of the host biological mechanism in which the epithelial cell get polarised to develop the affinity with the basement membrane of the cell. This results in an alternation of a biochemical process affecting the migratory dimensions of the host against the invasion of cancerous cells. CXCR̆4 gene is found effective in enhancing the signaling pathway of EMT resulting in metastasis [55]. Antibodies named as anti- CXCR̆4 inhibit the adhesion to the cancerous cell preventing its migration. *L. acidophilus* is found positive in down-regulation the expression of CXCR4 in mice as an animal model inhibiting colonic cancer [17,48,55].

Table. 1.6 EMT inhibition mechanism of the probiotics on the host cells

Sr.no	Probiotics	Cell line/host cell	EMT inhibition
1.	*L. acidophilus*	CT 26 cell line	The up-regulation of the apoptosis TNF-factor based on ligand TRAIL
2.	*L. casei*	HT 29 cell line	Inhibition of TRAIL regulated metastasis
3.	*L. casei*	CT 26 and HT 29 cell line	Inhibiting the proinflammatory cytokines over-expression
4.	*L. casei*	HT 29 cell line	Inhibiting the miR-221 expression

c) Inhibition of tumor microenvironment

The tumor microenvironment is developed by the communications bridging between the normal cells and tumor cells. These tumor-inducing cells are having the abilities to undergo various stages of the tumor formation called as tumorigenesis. These cells are non-malignant in nature and are related to the cells of the immune system and lymph nodal areas. The current investigation carried out by the researcher showed that Lactobacillus shows the anti-metastatic activities by altering the tumor microenvironment [56]. The animal model involving guinea pig demonstrated that use of *L. casei* YIT018 suppresses the lymph node metastases [48,57].

Table. 1.7 Mechanisms of tumor microenvironment inhibition by the probiotics [48,57,58]

Sr.no	Probiotics	Cell lines/host cells	Tumor microenvironment inhibition pathways
1.	*L. casei*	C47BL/6 mice	Suppresses tumor growth, protecting against pulmonary metastasis
2.	*L. casei*	C47BL/6 mice	Activation of natural killer cells as a cytolytic agent
3.	*Lactobacilli species*	C47BL/6 mice	Suppression of metastasis
4.	*Lactobacilli species YIT/ 9018*	Mice	Increase in IL-2 and IFN-γ level
5.	*L. brevis*	BALB/c mice	Decreases the liver metastasis originated from the metastatic breast carcinoma
6.	*L. brevis*	BALB/c mice	Increases the activity of the IFN-γ and IL-17 by activation of Natural killer cells.
7.	*L. casei 431 CRL*	Wister rat	Antitumor activity linked to CD4+ and CD8+ lymphocytes
8.	*L. casei* Shirota	HT 29 cell line	Activation of Natural killer cells
9.	*L. rhamnosus* GG	HT 29 cell line	Preventing the formation of the free radicals with enhancement of neutrophilic phagocytic activity

10.	*L. casei* Shirota	Caco 2 cell line	Down-regulation of the angiogenic IL-1β factor
11.	kefir	Caco 2 cell line	Inhibiting the proangiogenic factor IL-6

d) Inhibition of the cancer stem cells

The recent studies had showed that the appearance of the "cancer stem cells (csc)" as in a form of multi-potent candidature resulted in the malignancy conditions of the blood system. These cells show the abilities like the stem cells and get involved in the cellular damage in the form of metastasis [59]. These cells are demonstrating the heterogeneity in its functioning and showing the metastases specificity to each and every organ working in an organ system. The initiation and working of the csc are due to the integral signaling pathways demonstrated by these cells against the host immune system [60]. Alike stem cells, the self-transcriptional systems are generated by the crc resulting in induction of the hypoxia conditions in normal tissues. This further led to signaling interconnectivity development by the multipotency Oct4 pathway regulations. This initiates the metastatic cells to migrate from one organ to the other. *L. rhamnosus* down-regulated hypoxia 1α metastatic conditions showed by the *in-vitro* breast cancer and colonic cancer cell lines [48,59].

Table. 1.8 Cancer stem cells inhibition by the probiotics [48,61]

Sr.no	Probiotics	Cell line/host cell	EMT inhibition pathways
1.	*L. rhamnosus*	C47BL/6 mice	Inhibiting the (hypoxia-induced factor) HIF-1α signaling preventing metastasis
2.	*Bifidobacterium breve*	C47BL/6 mice	Suppression of tumor cells by inhibition of inflammatory cytokine mediators
3.	*L. plantarum*	HT 29 cell line	Activation of the Natural killer cells by inhibition of inflammatory cytokine mediators
4.	*L. crispatus*	HT 29 cell line	Down-regulation of HIF-1α signaling along with over-expression of *SFRP2*
5.	*L. casei*	HT 29 cell line	Activation of the Natural killer cells by inhibition of inflammatory cytokine mediators
6.	*L. bulgaricus*	HT 29 cell line	Activation of the Natural killer cells by inhibition of inflammatory cytokine mediators
7.	*Bifidobacterium infants*	C47BL/6 mice	Suppression of tumor cells by inhibition of inflammatory cytokine mediators

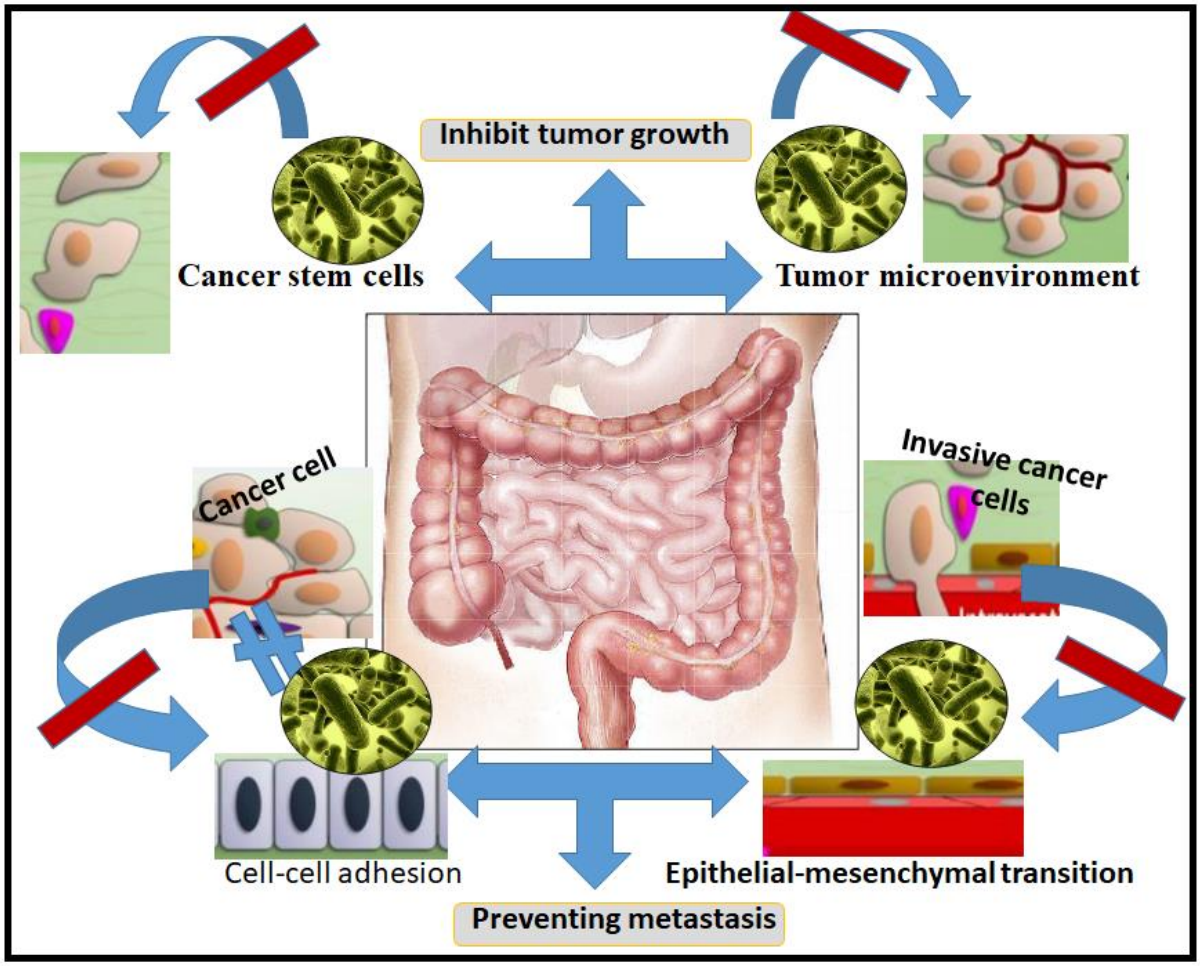


Fig. 1.5 Probiotics mechanism inhibiting the infestation of the cancer

1.5. Statement of problem

In this 21st century, many new synthetic drugs are been developed against cancer but come with one or many side effects. The cost of the new emerging drugs are higher and are beyond the range of the common people. These drugs are of synthetic origin and are given in different combinations producing the synergistic adverse effects on the host body. Each and every cancer is different and their line of treatment in this relationship is complex. Colorectal cancer is the third largest cancer in Asian countries after oral and breast cancer. Along with the diagnosis, the prognosis of the patient suffering from colorectal cancer is a critical issue.

Many of the functional food and nutraceutical formulations are available in the market but are limited to minor disease or disorders. Hence, to tackle this

problem there is the need for new novel nutraceutical formulation derived from the natural origin which can control the manifestation of these types of cancer and can be reproducible. Many probiotics formulations are widely available in the market for the treatment of bowel disorders. Lactobacillus are the genera mostly derived and isolated from the milk of the different milking animals. Furthermore, many of the reported cultures of the Lactobacillus are available as powder formulation possessing many biological activities like mucoadhesion. These properties of adhesion abilities to the intestinal lumen along with other properties such as hydrophobicity and auto-aggregation, maintain the proper bowel movement preventing disorders of the gastrointestinal tract. Many reported functional food has the ability to trigger and initiate an innate immune system of the host and biological properties, including antimicrobial properties, which combat the activities of pathogenic microorganism. The abilities possessed by some probiotic strains to scavenge the free radicals, work as natural antioxidant agents contributes as an anticancer mediator. The prepared formulations are ideal if they mimic as the natural antioxidant mediators and comes with long shelf life. The use of probiotics formulations in an existing market is low, the reason behind this problem is the short shelf life of the product and its need for refrigeration. Thus, the Lactobacillus formulation should contain longer shelf life along with the surplus biological activities like antimicrobial, antioxidant and anticancer activities. In today's market, no any formulations of functional food origin are available with important biological properties, hence we are proposing a new innovative functional food as granule formulations. In this technique, the newly isolated strain of Lactobacillus is grown in the milk of milking animals. The fermented products will be formulated into the granule formulations with the long-term shelf life. The main aim of the research is to formulate granule formulations with the properties of muco-adhesion to the site of cancerous cells. As these attached friendly microbes may heal the cancer site more effectively by antioxidant activity and help in triggering innate immunity system along with the release of certain chemicals (proteins) and killing the cancer cells.

Based upon these parameters the objective of the current studies deals with-

a) Isolation of probiotics strain and its development in a natural medium
b) *In-vitro* gastrointestinal simulation studies
c) Flourishing of the isolates in the milk of different milking animals
d) Batch optimization using proper polymer via spray drying and lyophilisation methods
e) *In-vitro* antioxidant and supportive antagonistic studies of generated granules
f) *In-vitro* colon cancer cell line cytotoxicity and anti-colon cancer activity of isolates
g) *In-vivo* antioxidant and anti-colon cancer activity of isolates

The entire research work is focused mainly into 7 sections; the first section of the work is estranged on the isolation of a new strain of the Lactobacillus species from the sheep milk along with its molecular identification. The second section of the work is estranged on exploring the viability and nature of Lactobacillus species in simulated stress condition of the gastrointestinal tract. As many Lactobacillus strains are discovered till date, but few strains are available with the stress tolerance bearing abilities. The third section of the work involves the flourishing of isolates in the milk as natural media along with the optimization of the batch. It is a plan to use milk of different local milking animals and to investigate the optimal growth in form of colony forming unit (cfu). The fourth section of the work is estranged on the development of the granule formulations by drying techniques such as spray dry and lyophilization methods. It involves the process of microbial growth optimization in relation to long-term shelf life and integral viability. The fifth section of the work involves the screening of the *in-vitro* antioxidant potential of the isolates along with supportive antagonistic activity involving auto-aggregation and hydrophobicity studies. The sixth section of the study deals with the *in-vitro* colon cancer cell line cytotoxicity and anti-colon cancer activity of isolates using HCT 115 cell lines. The last part deals with *in-vivo* anti-colon cancer activities on male wistar rats induced by the chemical agents.

Thus, the major goal of the study is to isolate new probiotics microbes from natural sources along with an evaluation of its nature and nomenclature. The second major challenge is to generate the formulations possesses the biological properties of antimicrobial, antioxidant and anticancer activities with long term shelf life. The initial all the testing will be done using the HCT 115 cell line and further, we can test pharmacological activities on male wistar rats by the approval of the Institutional animal ethical committee (IAEC).

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Chapter 2

The part of this chapter have been published as a review article

Milk as "Pre and probiotics" : A theoretical background

Article published as Cover page

Health benefits of Probiotics by antioxidant activity: A review

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Abstract:

2.1 Introduction

The human gastrointestinal tract includes complex microbiome or microbial genome present since birth located in different anatomical parts [1]. The symbiotic or commensal relationships are being developed between the host organism and microbiota. The composition of this microbial load is beneficial to host as it helps in digestion of food and maintaining immunological functions and are called probiotics [2]. These helpful bacteria are specifically found in the anaerobic colonic conditions in large concentration as an integral part of the host digestive system.

The types, groups of carbohydrates consumption and nature of diet in human generally alter the growth and activity of intestinal microflora. These undigested residual part on which colonic microflora thrive and dwells on it are called as prebiotics [3]. The consumption of prebiotics not only promote the growth of probiotics but also equally contribute in improving the digestive system of a host; downregulating allergic response, improving colonic microenvironment and decreasing the intestinal diseases and infections. Prebiotics is also found important in mineral absorption at a colonic region which includes assimilations of iron, calcium, and zinc [4–6].

2.2 Origin of prebiotics from plant sources

The plants, seeds, vegetables or fruits rich in inulin, fructooligosaccharides (FOS) are the basic sources of prebiotics [7,8]. The plant's resources belonging to raffinose family are rich in FOS because they not get absorbed in the intestine and regulates the growth of probiotics in the human gut.

Many ingestible fibers obtained from *Legnaria siceraria, Cucurbita maxima* and *Barassus hispida* are found as a rich source of prebiotics [9]. Prebiotics are also found in fruits like *Artocarpus heterophyllus* (jackfruit), *Barassus flabellifer* (palm fruit), root crops like *Dioscorea esculenra* (gembili) and in mushroom-like *Agaricus bisporus* [10]. The genetic variability in prebiotics carbohydrates concentration was observed in *Hardeum vulgare* (barley), *Cicer arictinum*

(chickpea), *triticum aestivum* (wheat) and *Abelmoschus esculentus* (okra) [11]. The soluble dietary fiber (sdf), insoluble dietary fiber (idf) and total dietary fiber (tdf) are the important component of prebiotics which promotes proliferation of the probiotics in the host gastrointestinal tract [12].

Table 2.1. Prebiotics contents from the plant sources

Sr. no.	Cereals and roots	Reported prebiotics constitutes [5-15]
1	Barley	Grain fructan is present as 1.0 to 4.0 g per 100 g
2	Lentil	Sorbitol is present as 1001 to 1311 mg per 100 g; mannitol, stachyose, and raffinose (in traces)
3	Durum wheat	Insoluble dietary fiber, soluble dietary fiber, and total dietary fiber are present approximately as 150 g per kg of the dry weight
4	Lentil	Raffinose, verbascose, and stachyose are present approximately as 1.4 g, 2.8 g and 1.5 g per 100 g
5	Wheat	Grain fructan is present as 0.5 to 2.1% of dry weight
6	Pears	Total dietary fiber content is 3.1 g per 100 g of fresh weight
7	Strawberry	Total dietary fiber content is 2 g per 100 g of fresh weight and very less calorific value
9	Avocado	Total dietary fiber content is 2 g per 100 g of fresh weight and rich in minerals and vitamins
10	Apples	Total dietary fiber content is 2 g per 100 g of fresh weight

11	Banana	Insoluble dietary fiber is present as 2.6 g per 100 g of fresh weight
12	Onion	Fructan contents are present as 0.84 to 3.04%
13	Watermelon	High fructan contents are present as 0.4 g per 100 g and are rich in mineral contents

In-vitro studies showed that sdf obtained from Khorasana (ancient durum wheat) promotes the growth of *L. plantarum* [8]. Sweet wheat is found rich in fructan and sdf which is two to three times greater than (*Triticum monococcum*) wheat [11]. Grain fructan obtained from the rye in the form of tdf is found in concentration 3.8 to 4.0 g per 100 g [16]. The researcher found that resistant starches are converted into the short chain fatty acid chain (scfas) by probiotics thus promoting various health benefits. Grain legumes rich in lupin and chickpea derived fibers are found to enhance the growth of Bifidobacteria in the colonic region [13]. Lentils are found abundant in prebiotics carbohydrates along with raffinose, sorbitol, and stachyose promoting the growth of the lactic acid bacteria. The other plants which are found rich in prebiotics properties include *Gigantochloa levis* (Bamboo) and seeds of *Amygdalus communis* (almond) which are rich in polysaccharides promoting microbial growth in the human gut [17,18].

Prebiotics indirectly along with the probiotics is found to promote health benefits. Regular intake of dietary fibers is found to reduce the risk of cardiovascular diseases along with gastrointestinal disorders. The undigested fibers from the small intestine are found to get assimilated into the colonic region in form of scfas. Obese and lien conditions are found to get altered by high intake of fibers, as this changed the microbiota composition profile in the host by degrading extracellular fat [12].

2.3 Milk as prebiotics

Milk is considered as an essential functional food in the human diet. Milk is the basic source of food not only to the human beings but also to the probiotics for their growth and development thus it acts as prebiotics source [19]. The reported literature showed and proved that the basic milk components are water, carbohydrate, fat, protein, vitamins, minerals, minor biological proteins and enzymes which vary from type of milking animals and its breed. The energy value of milk is calculated in the form of fat contents in dry matter [20]. The reported data showed the sheep milk with the highest energy ± 5900 kJ/kg, compared to cow milk ± 3100 to 3700 kJ/kg, buffalo milk ± 3450 kJ/kg, camel milk ± 3283 kJ/kg, and goat milk ± 3018 kJ/kg [21]. The main components of milk with its nutritional value is proteins. Milk proteins used in dairy industries are categorized as casein content and whey protein components. The nutrient content of the milk of different animals are as follows [21,22]:

Table 2.2. The nutrient contents of milk in different milking animals

Milking animal	Protein (%)	Fat (%)	Lactose (%)
Cow (*Bos taurus*)	3.42	4.09	4.82
Buffalo (*Bubalus bubalis*)	4.38	7.73	4.79
Sheep (*Ovis aries*)	5.73	6.99	4.75
Goat (*Capra hircus*)	3.26	4.07	4.51
Camel (*Camelus)*	3.26	3.80	4.30
Donkey (*Equus asinus*)	1.59	0.28	6.73

The mineral contents observed in the milk of different milking animals include calcium, phosphorus, sodium, calcium, phosphorus, iron, iodine potassium, chloride, iodine, and magnesium. The nutritive value of the milk depends upon the

mineral contents and its bio-viability to host after its consumption. The mineral contents from different milking animal milk include [23]:

Table 2.3. The mineral contents of milk of different milking animals

	Cow	Buffalo	Sheep	Goat	Donkey
Calcium (mg/100 g)	122	112	195	134	67
Phosphorus (mg/100 g)	119	99	125	121	48
Potassium (mg/100 g)	152	92	136	181	49
Magnesium (mg/100 g)	12	8	18	16	3
Sodium (mg/100 g)	58	35	44	41	21
Iron (μg/100 g)	80	161	72	7	-
Zinc (μg/100 g)	530	410	520	56	-
Iodine (μg/100 g)	2.1	-	10.4	2.2	-

Selenium (μg/100 g)	0.96	-	3.1	1.33	-

Similarly, the milk also is considered as a valuable resource of many vitamins i.e. both fat and water-soluble ones. Thus, the concentration of vitamins in the different milking animals are as follows [20,23,24]

Table 2.4. Vitamins contents in the milk of different milking animals

	Cow	Buffalo	Goat	Sheep
Vitamin A (μg)	126	129	185	146
Vitamin D (μg)	2	-	2.3	1.18
Thiamin (mg)	0.05	0.127	0.07	0.08
Riboflavin (mg)	0.16	0.33	0.21	0.38
Niacin (mg)	0.08	0.22	0.27	0.41
Vitamin B_6 (mg)	0.045	0.05	0.046	0.08
Folic acid (μg)	1	15	1	5
Vitamin B_{12} (μg)	0.07	0.88	0.06	0.72

Milk is considered an essential functional food in the human diet. The major source of milk in India, especially in Maharashtra, is milking from buffalo. Along

with the quantity, quality of milk, with its contents and compositions makes a lot of impact in dairy industries. The commonly domesticated buffalo in Maharashtra are Bhadawari, Mehsana, and Nagpuri for their milk. The nutrient contents in the milk of all the milking animals include the fat content, total solid content, vitamins, minerals, lactic acid bacteria, proteins, and non-protein contents [25]. The detailed illustration of the same is presented in the chart given below.

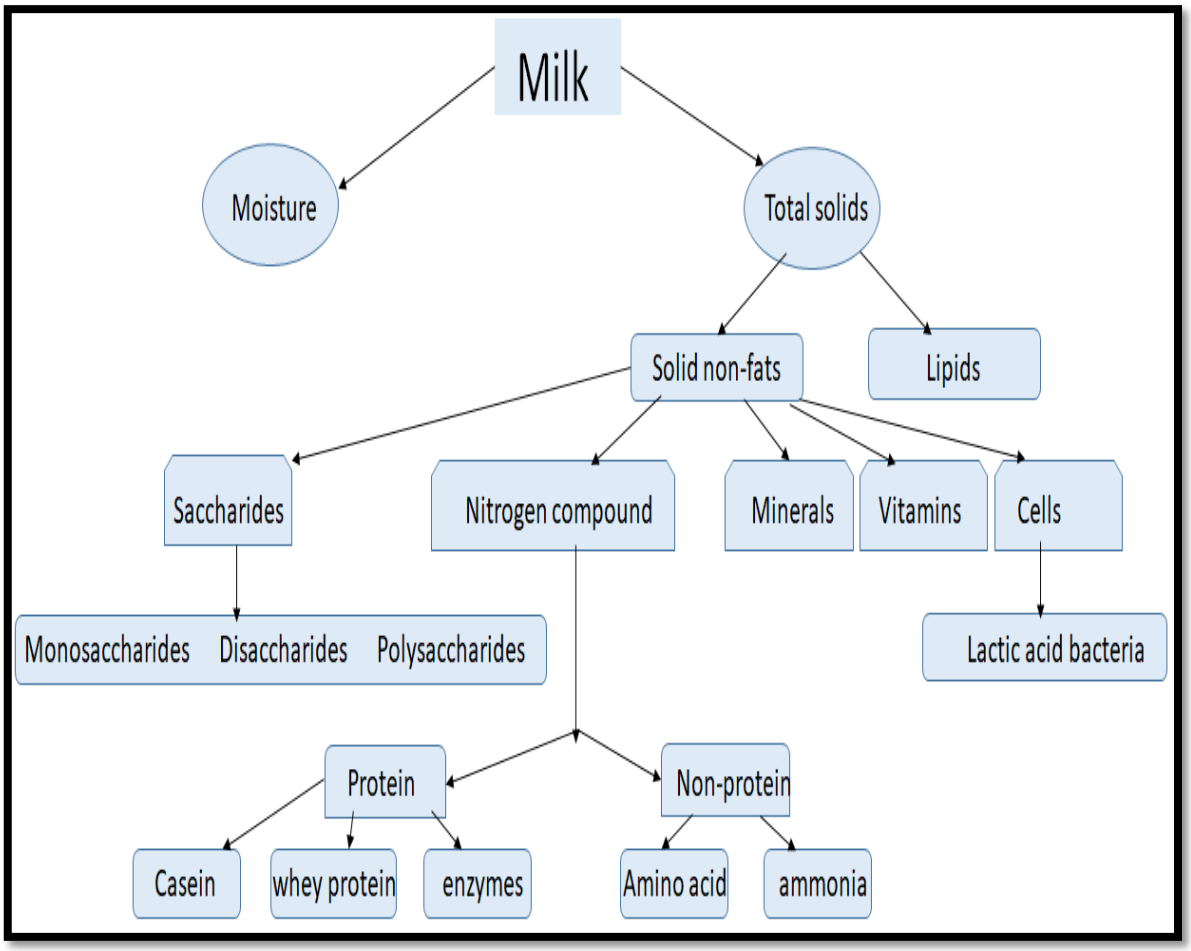

Fig 2.1. The general composition of the milk and its content

Milk oligosaccharides, which are one of the vital components of milk are found with the non-infective properties against pathogenic microbes. The domestic animal's milk contains large amounts of sialyloligosaccharides and neutral oligosaccharides. The colostrum thus can act as ideal candidature raw material for the large-scale production of anti-infective oligosaccharides [26,27]. The milk oligosaccharides may act as prebiotics. Many reported literature demonstrated that the growth of Bifidobacterium in the infant's colon are probiotics in nature [28,29]. The

mechanism involved is the lowering of the colonic pH thus inhibiting the growth of pathogenic microorganisms and promoting the growth of lactic acid bacteria.

The milk containing colostrum is found rich in sialyloligosaccharides [30]. This carbohydrate acts as prophylaxis mediators and prevents the infants or person's consuming the milk against the pathogenic microbes. They also promote the growth of colonic microflora and enhances innate immunity, along with the nutritional benefits. As the carbohydrate sources, these glycoconjugates are found with the significant biological functions, thus various attempts are been made to biosynthesis it from milk of domestic animals [26]. The milk, obtained after parturition contains the highest percentage of colostrum may be used for the pilot to large-scale manufacturing of milk oligosaccharides [31]. The different types of oligosaccharides obtained from the milk of domestic animals include:

Table 2.5. The oligosaccharides obtained from the milk and its antimicrobial activities

Oligosaccharides (abbreviation) [17,18,32]	Basic structure	Concentration in milk (mg/l)	Inhibition of Pathogenic strain
a-3'-galactosyl-lactose (a-3'GL)	Gal(a1-3)Gal(b1-4)Glc	30–50	
3'-galactosyllactose (b-3'GL)	Gal(b1-4)Gal(b1-4)Glc	36.25	*L. monocytogenes*
6'-galactosyllactose (b6'GL)	Gal(b1-6)Gal(b1-4)Glc	48.5	*L. monocytogenes*

3'-N acetylneuraminyllactose (3'-SL)	Neu5Ac(a2-3)Gal (b1-4)Glc	30–50	*H.pylori*, *P. aeruginosa*, Influenza virus
6'-N-acetylneuraminyllactose (6'-SL)	Neu5Ac(a2-6)Gal (b1-4)Glc	50–70	*P. aeruginosa*, *Influenza virus*
6'-N-glycolylneuraminyllactose	Neu5Gc(a2-6)Gal (b1-4)Glc	40–60	-
Diasylyl-Lactose (DSL)	Neu5Ac(a2-8)-	1–5	-
N-acetylglucosaminyl-lactose (NAL)	GlcNAc-(b1-6)Gal (b1-4)Glc	20–40	-
Glycolyl-neuramyl-lactosamine (New)	-	-	-
N-acetyl-glucosaminyl-hexosyl-lactose (NAHL)	Gal-(b1-4)-GlcNAc-(b1-6)Gal-(b1-4)Glc	Trace amount	-

N-Di-N-acetyl-glucosaminyl-lactose (DNAL)	-	Trace amount	-
3'-Sialyl-6'-galactosyl-lactose (3-SHL)	Gal-(b16)Gal(b1-4)Glc ↑NeuAc(a2-3)	Trace amount	-
6'-Sialyl-6'-galactosyl-lactose (6-SHL)	Gal-(b1-3)Gal(b1-4)Glc ↑NeuAc(a2-6)	Trace amount	-

2.4 Probiotics and its classification

The advanced research focusing on the study of the microbiota is that of the human gut microbiome and its application. The human microbiome project (HMP), and the metagenomics study of the human intestine are dealing with the wide range of microbiota investigation and their role in the gastrointestinal tract [33]. The adhering ability of the probiotics to the intestinal of the host and the ability to assimilate the undigested prebiotics, is the basic research areas of focus for the researchers' [34].

Gut microflora is the main source of the live probiotics [35]. The genera and species of the microorganism used as probiotics are *Streptococcus*, *Lactobacillus*, *Enterococcus*, *Bacillus*, *Bifidobacterium*, *Pediococcus*, *Propionibacterium*, *Leuconostoc*, *Candida pintolopesii*, *Saccharomyces cerevisiae*, *etc.* Lactic acid bacteria such as *Lactobacillus* (LAB) and *Bifidobacterium* are present in the

human gastrointestinal tract since birth [36]. The microbiota present in the intestine and their mechanism of action with the cfu count is as follows:

Table 2.6. Intestinal microbiota and their mechanism of the actions

Gastrointestinal tract regions	Microbiota load in cfu per gram	Mechanism [37,38]
Stomach and parts of the duodenum	$< 10^3$	Hydrochloric acid in liquid and gaseous form along with peristalsis movement and bile activity prevents the adhesion and colonization of the bacteria
Ileus, distal ileum region	10^2–10^3, Microaerophilic condition	Fermentation of carbohydrates (prebiotics) by lactic acid bacteria
Large intestine (especially colon)	10^{10}–10^{12} anaerobes conditions	Based on the location of most microbiota in the specific areas of the host's gut

The strains that are part of milk or dairy products are called as a conventional source of probiotics [39]. These include microbes such as *B. cereus* var. *toyoi, B. coagulans, B. racemilacticus, B. lichenformis, B. clausii, B. laterosporus,* and *B. pumilus*. The probiotics isolated from the non-intestinal source, vegetables, fruits, traditional non-dairy fermented foods, and drinks are called non-conventional probiotics [40]. The non-conventional sources of probiotics were evaluated for

potential probiotics properties and used as functional food against many diseases and disorders. Due to the health benefits, these probiotics strains are rationally used as commercial functional food based on the following criteria as: (1) source and identification of live bacteria along with its functional characterizations (2) dose of bacterial administration that also based on its type of strains or combination with different species or with other prebiotics source etc., (3) functional benefits to the host along with its safety of use as per the concept of "generally recognized as safe" (GRAS) for food additives and substances [41].

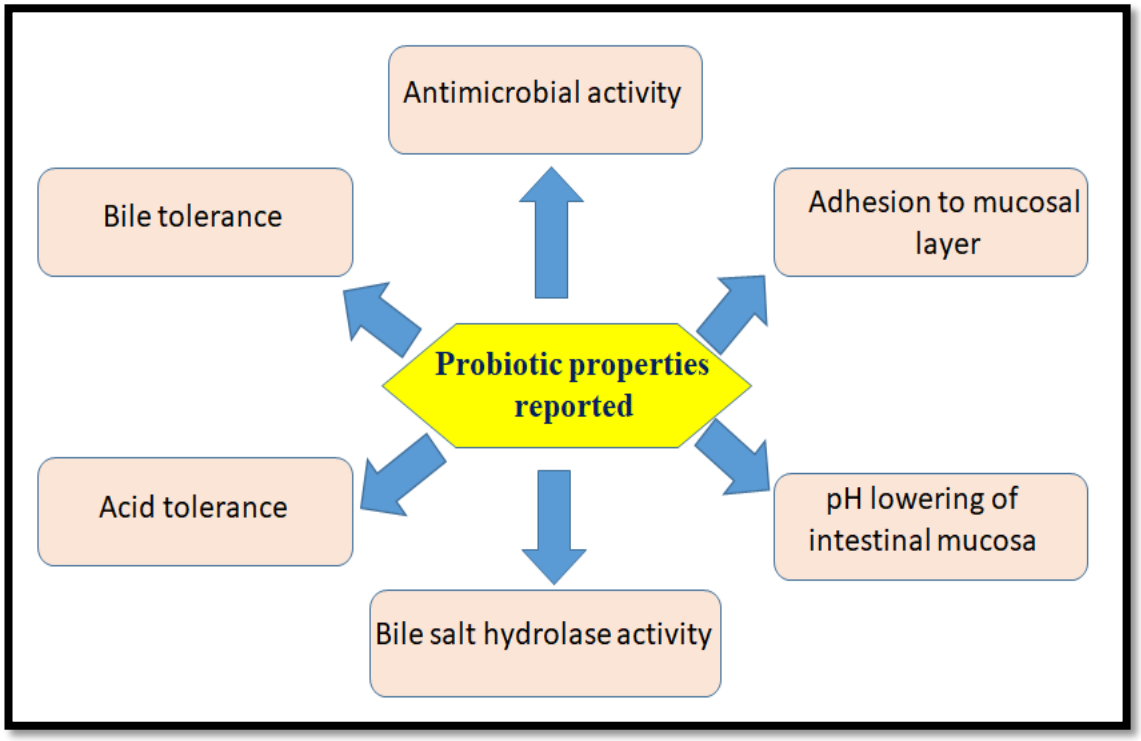

Fig 2.2. Reported probiotics properties

Probiotics microorganisms *Lactobacillus plantarum* have been isolated from the citrus fruits whereas *Leuconostoc mesenteroides* was isolated from the tomatoes pulp [42]. These isolated strains produced a bacteriocin-like substance with the ability to tolerate low pH, bile activity with the strong antimicrobial activity against enterobacteria [43]. The LAB from the fruits and vegetables like a dragon, papaya, ginger, watermelon, and guava produces chemicals like hydrogen peroxide, bacteriocin, lactic and propionic acids [29]. The LAB isolated from the honey was *Lactobacillus* spp., mainly *Lactobacillus kunkeei* showing the

antagonistic effects preventing its spoilage by hindering the growth of yeast [44]. In the case of the fermented non-dairy food, it includes either from plant or animals sources. The fermented plant materials mostly contain *L. plantarum* and *L. rhamnosus* which grew under (6%) NaCl concentrations [45]. The Thai traditional salted crab (*Poo-Khem*) contains *L. plantarum*, *Enterococcus thailandensis* and *L. fermentum* exhibiting probiotic properties [39]. Many traditional Thai foods contained the LAB and *Pediococcus pentosaceous* which were obtained from the fermented pork with the ability of acidic conditions tolerance of pH ranged from 1-2. Various strains such as *L. pentosus, L. plantarum*, and *L. paracasei* were isolated from the green olives [46]. The fermented Croatian sausages were found with the *L. plantarum* and *L. brevis* showing antioxidant activities [47]. The traditional food idli prepared by fermentation of rice was found to contain the *L. plantarum* showing the co-aggregation properties against pathogens like *Escherichia coli* and *Listeria monocytogenes* [34].

The Turkish fermented drink 'Boza' was found to contain the *Lactobacillus plantarum* as a probiotic microbe producing organic acids against the pathogenic bacteria *Bacillus cereus and Salmonella typhimurium* [48]. Similarly, the Nigerian traditional drink called *Kunu-Zaki* prepared from sorghum and cereal grains contained the *Lactobacillus along with Pediococcus* and *Lactococcus* species [49]. Many of the *Lactobacillus strains such as L. salivarius* obtained from the nose of dolphin were found to inhibit the growth of pathogenic *Salmonella* strains [50]. *Leuconostoc mesenteroides* strains were reported to be isolated from the fishes such as snakehead and Nile tilapia [42]. The immune stimulation and antimicrobial activities showed by the *B. pumilus* and *B. clausii* were isolated from the *Epinephelus coioides* fishes [51]. The probiotics *Leuconostoc cremoris* was isolated from salmon fish showing the non-specific immune response when administrated in the host. Similarly, the *Lactobacillus paracasei* isolated from shellfish showed antimicrobial activities against *E. coli* and *Bacillus cereus* along with acid and bile tolerance [52]. In general, the probiotics are classified as Lactobacillus, *Bifidobacterium and other lactic acid bacteria* [36].

Table 2.7. Different genera of the probiotics and its general classification

Sr.no	*Lactobacillus strains*	*Bifidobacterium strains*	Other lactic acid bacteria [36]
1	*L. acidophilus*	*B. adolescentis*	*Leuconostoc mesenteroides*
2	*L. casei*	*B. infantis*	*Enterococcus faecalis*
3	*L. crispatus*	*B. lactis*	*Lactococcus lactis*
4	*L. gallinarum*	*B. longum*	*Streptococcus thermophilus*
5	*L. gasseri*	*B. animalis*	*Sporolactobacillus inulinus*
6	*L. johnsonii*	*B. bifidum*	*E. faecium*
7	*L. paracasei*	*B. breve*	*Pediococcus acidilactici*
8	*L. plantarum*	-	-
9	*L. reuteri*	-	-
10	*L. rhamnosus*	-	-

The non-lactic acid bacteria are named as *Bacillus cereus var.* toyo*i Escherichia coli* strain nissle, *Propionibacterium freudenreichii* , *Saccharomyces cerevisiae, S. boulardii* etc [5].

The probiotics show the antimicrobial activity due to the release of the different chemical components. As per the reported literature, *Lactobacillus* GG produces a wide spectrum of natural antibiotics to prevent the growth of the pathogens. Similarly, *L. acidophilus* releases extra-proteinous

materials such as acidophilin, acidolin, lactocin B, lactocidin [53]. The antimicrobial component derived from the *L. delbrueckii* ssp. bulgaricus is Bulgarican inhibiting the growth of enterobacteria. Lactolin is released as the exoproteins by *L. plantarum* which acts as an auto-aggregation agent preventing the adherence of the pathogenic strains to the intestinal lumen [54]. Lactobacillin and lactobrevin are the two antimicrobial agents released by *L. brevis* showing the antioxidant activity [55]. *L. reuteri* releases the reuterin which acts as a potent water-soluble antimicrobial multi-compound inhibiting the growth of both Gram-positive and Gram-negative bacteria, along with protozoa and yeasts. *L. sake* produces heat-stable polypeptide called as sakacin A showing bacteriostatic activity [56]. *L. johnsonii* produces antimicrobial peptides lactocin F used in many nutraceutical formulations in the treatment of vaginal bacterial infections [57]. *L. helveticus* releases the antimicrobial compound hilveticin J which is active at neutral pH under both aerobic or anaerobic conditions and thermo-labile (30 min at 100 ^{o}C) [58]. Similarly, the proteins such as nisin, lactostrepsin, lactocin, lacticin are being released by the *Lactococcus lactis*; Pediocin by *Pediococcus pentosaceous* and streptophilin is released by *S. thermophilus* showing the antimicrobial activities [53,54]. Based on the commercial application and discovery by the company/ University the probiotics strains and their sub-strains are named as follows:

Table 2.8. Newly isolated probiotics strain by company and Universities

Commercial Strains [39 45]	Sub-strain	Company/ University
L. acidophilus, L. paracasei, B. lactis	As LA-1, CRL 431 and Bb-12 respectively	Horsholm, Denmark
L. casei *B. breve* strain Yakult	As Shirota	Yakult, Japan
L. acidophilus, B. longum	As SBT-2062 and SBT-2928	Snow Brand, Japan

L. acidophilus, L. rhamnosus	As R0011 and R0052	Institute Rosell, Canada
L. acidophilus	As NCFM	Rhodia, West Indies
L. acidophilus	As DDS-1	Nebraska Cultures, Lincoln
L. casei (Immunitas)	As DN014001	Danone Le Plessis, France
L. fermentum, L. rhamnosus	As RC-14 and GR-1	Urex Biotech Inc. Ontario
L. johnsonii	As La1	Nestle, Switzerland
L. plantarum, L. Rhamnosus	As 299V, 271	Probi AB Sweden
L. reuteri	As SD2112	BioGaia, Raleigh
L. rhamnosus	As GG	Valio Dairy, Finland
L. rhamnosus	As LB21	Essum, Sweden
Lactococcus lactis L. salivarius	As L1A, UCC118	University of Ireland
B. longum	As BB536	Morinaga Milk Industry Japan
L. delbrueckii subsp.	As *bulgaricus* 2038	Meiji Milk Products, Japan
L. acidophilus	As LB	Lacteol , France

L. paracasei	As F19	Arla Dairy, Sweden
L. crispatus, L. casei	As *CTV05, DN 114*	Gynelogix, Boulder, France
B. lactis	As HN019	New Zealand Dairy Association
L. plantarum, ***L. rhamnosus***	**As ARJD, JDARSH**	**D Y Patil University, Kolhapur, India**

2.4.1 Health benefits of probiotics

The probiotics mostly act as a functional food, not only as an antimicrobial agent but also as antioxidant mediators. Due to the biological origin and probiotics properties, these microbes are showing health benefits and healing many diseases and disorders.

1) Resistance to intestinal pathogenic bacteria [10,37,59,60,61]

Various mechanism is showed by the probiotics in preventing the manifestation of the intestinal pathogenic bacteria. Mostly *L. plantarum* showed the antagonism activity against the *E. coli* and *Streptococcal* infection. They also increase the innate immunity system by triggering the antibody formation against the intestinal pathogens such as *E. coli,* to prevent its colonization. The Lactobacillus genera containing bacteria such as *L. acidophilus, L. delbrueckii, L. helveticus, L. salivarius* etc., releases the chemicals such as bacteriocins and lactocins which are the antimicrobial peptides inhibiting the growth of pathogens. Probiotics reduce the symptomatic conditions of the infectious diarrheal by relieving the acute gastroenteritis. The other mechanism involved is the lowering of the pH in the colonic region, inhibiting the adhesion of the pathogenic strains to the intestinal lumen. Auto-aggregation and co-aggregation are the two other mechanisms

inhibiting the colonization of pathogens. The hydrophobicity of the host-probiotics interaction also prevents various diarrheal diseases.

2) Lactose digestion [62]

Lactose intolerance is the basic problem observed in the patients as they are unable to digest the milk lactose. *L. acidophilus* is a strain of bacteria that is found to digest lactose and helps to treat the person suffering from the lactose intolerance. In this, the bacteria release the enzyme lactase which acts on lactose and helps in its assimilation.

3) Controlling the bacterial overgrowth in the small bowel [37,63]

Due to the anaerobic condition, the overgrowth of the pathogenic bacteria generally takes place in the colonic region. The Lactobacillus genera are found effective to adapt in the microaerophilic conditions preventing the toxic metabolite production of the enterobacteria preventing dysbiosis conditions.

4) Immune modulatory activity [2,64,65]

Some Lactobacillus microbes get into the Peyer's patches and show immunomodulatory action by inducing the secretion of cytokines. Few LAB strain induces dendritic cells (dc) maturation, stimulating the secretion of Interleukins-10 and Interleukins-12 as a part of adaptive innate immunity. The proper pharmacological dosing with the LAB stimulates not only B (humoral immunity) but also T lymphocytes (cell-mediated immunity) along with the phagocytic component activations. Recent studies showed that LAB is with the potential in preventing the bronchial mucosa to secrete IgA-secreting cells in adverse respiratory infections. Some probiotics prevent the inflammatory response of the intestinal immune system by anti-apoptotic mechanism preventing the colitis-like condition. Some of the LAB are able to secrete the mucus content, increasing the activity of natural killer cells.

5) Allergic conditions [65,66]

Probiotics mostly have the protein binding affinity by means of hydrophobicity, preventing the translocation of the antigen into the blood system from the gut or respiratory tract. This prevents allergic responses and anaphylaxis conditions. It also prevents the immunological responses arises by triggering of mast cells to release the histamine from intestinal lumen caused by the antigen stimulations.

6) Cardiac disorders [67]

The increase in obesity is the major reason for hypertension and heart diseases. The probiotics are found with the abilities to combat the cholesterol level by assimilating the excessive fat by its metabolism. LAB generally helps in alteration of the bile salt hydrolase along with the anti-oxidant activity digesting the fat and preventing the blood vessel blockage and heart attacks.

7) Urogenital Infections [68]

LAB generally get adhered to the urinary and vaginal mucosal cells with the auto-aggregation mechanism and inhibit the growth of pathogenic strains. The antioxidant mechanism is seen in case of the *Lactobacillus iners and Lactobacillus crispatus* inhibiting the growth of pathogens by the release of H_2O_2 in the vaginal mucosa.

8) Necrotizing colitis [69]

The intestinal wall of the infants is invaded by the pathogenic bacteria resulting in the infection and inflammatory conditions with the disruption of the bowel. The recent study showed that dosing of *L. reuteri* prevents the severity of necrotizing colitis condition in infants at the early stage.

9) Caries gingivitis [70]

Caries gingivitis conditions arise by the colonization of S*treptococcus mutants* in the buccal cavity, especially near the teeth surface. The recent studies showed that the mucosal wash by *L. reuteri* as a mouthwash or the use of heat-killed LAB prevents enamel caries. This therapy can be used as a prophylactic measure to prevent gingivitis conditions.

10) Cofactor for vitamin synthesis [24] [71]

Lactobacillus are auxotrophic in nature in case of vitamins production. Several strains of the LAB are found with the capability to synthesize riboflavin and vitamin B_{12} type of water-soluble vitamins. Thus, the large-scale production of the vitamins by organic methodology became easy by the use of LAB and fermenting them at the manufacturing level.

The use of various types of probiotics against the numerous health disorders are demonstrated as [72, 73, 74, 32,38,57,75, 76]

Table 2.9. Probiotics used against different diseases

Sr. no	Types of disorder	Probiotics for treatment
1	Acute diarrhoeal infections in children	*L. rhamnosus GG*
		L. reuteri 55730
		S. boulardii
2	Acute diarrhoeal infectious in adult	*Enterococcus faecium*
3	Prophylaxis of antibiotic-associated diarrhea	*S. cerevisiae*
		L. rhamnosus GG

		B. lactis Bb12 + S. thermophilus
		Enterococcus faecium
		L. casei in skimmed milk along with *L. bulgaricus* + *S. thermophilus*
		B. clausii
		L. acidophilus + L. casei
4	Prophylaxis of rotavirus nosocomial infection	*L. rhamnosus*
		S. thermophilus + B. lactis
		B. lactis
		L. reuteri
5	Prophylaxis of *C. difficile* infection	*L. rhamnosus*
		S. thermophilus + B. lactis
		L. reuteri
6	The adjuvant in therapies for Helicobacter pylori Eradication	*L. rhamnosus GG*
		B. clausii
		S. boulardii
		L. casei + S. thermophiles
7		*B. infantis*

	Reduction of irritable bowel syndrome symptoms	*L. rhamnosus GG*
		B. longum, B. breve
		L. delbrueckii subsp.
		L. acidophilus,
		subsp. shermanii JS
		B. infantis
		L. plantarum
		L. casei
8	Prevention against the remission of ulcerative colitis	*E. coli Nissle 1917*
9	Prevention against the remission of pouchitis	*B. longum*
		B. infantis
		L. acidophilus
		L. delbrueckii subsp
		S. salivarius subsp thermophilus
		L. plantarum
10	Prevention of necrotizing condition of the colon	*L. acidophilus*
		L. delbrueckii subsp

		S. salivarius subsp thermophilus
		L. acidophilus + B. infantis

2.4.2 Role of probiotics in cancer prevention and treatments

Probiotics are found effective in the different types of cancer treatment. Probiotics as whole microbes or their proteins, enzymes are found as effective nutraceutical agents or mediators healing stress condition of the body, relieving and preventing the cancer formation in the host [77].

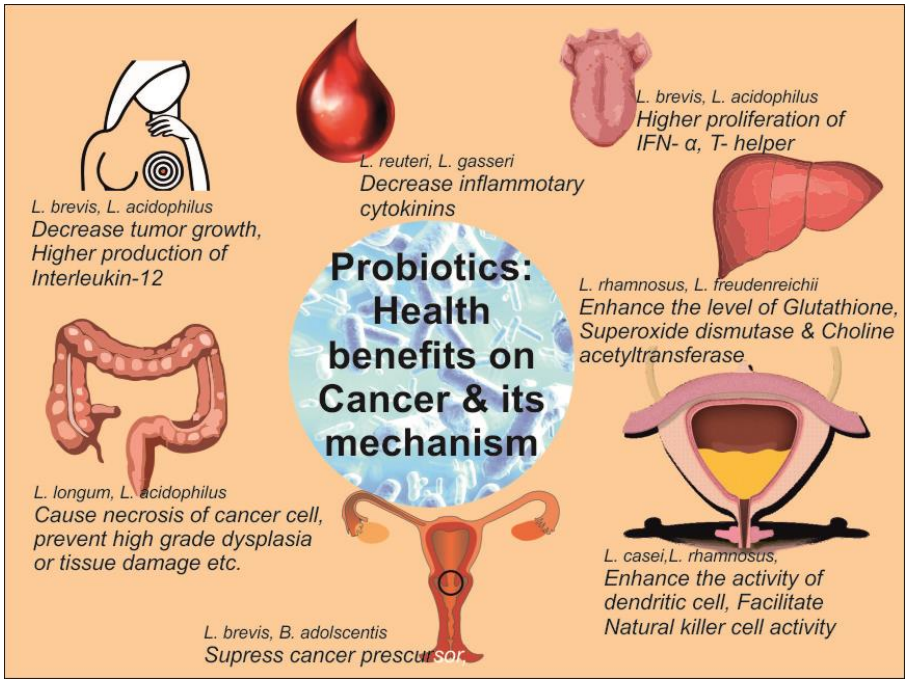

Fig 2.3. Mechanism and health benefits of probiotics against cancer

The anticancer nature of the probiotics can be divided into various sub-types based upon its vast research areas and techniques used. The study can be divided as cell line (*in-vitro/in-situ*) studies, animal models and clinical trials.

2.4.2.1 *In-vitro/In-situ* cell lines study [78–84]

The antiproliferative, antioxidant and anticancer nature of the probiotics has been demonstrated successfully on gastric cancer and colonic cancer cell lines. Many researchers reported that the lysate or supernatant of Lactobacillus group showed antitumor activities in *in-vitro* cytotoxicity study model. *Lactobacillus rhamnosus* GG proven to show the concentration-dependent anticancer activity on both gastric and colonic cancer cells especially on HTC 29, while *Bifidobacterium adolescentis* inhibited the growth of the HT 29, SW 480, and Caco 2 cell line [78]. Similar activities were reported in case of *Bifidobacterium animalis, Bacillus lacti, and Lactobacillus acidophilus,* etc. On the similar grounds the anticancer model developed by using the different cell lines and their activities reports are as follows [78–91]:

Table 2.10. *In-vitro* cell line studies using different probiotics

Probiotic formulations	Colon	Stomach	Breast	Liver	Cervical
L. reuteri	HCT-116, DLD-1	Human gastric adenocarcinoma epithelial cell line (AGS) cells	DN	DN	DN
L. kefiri	HT-29	Human gastric adenocarcinoma epithelial cell line (AGS) cells	DN	DN	DN
L. casei	CT-26, HT-29, WiDr, DLD-	Human gastric adenocarcinoma epithelial cell line (AGS) cells,	DN	DN	DN

	1 and. CX-1 cells	HGT-1			
B. adolescentis	DN	DN	DN	DN	DN
L. acidophilus	HT-29, WiDr, DLD-1, and CX-1 cells	Human gastric adenocarcinoma epithelial cell line (AGS) cells	MCF-7	DN	DN
L. bulgaricus	DN	Human gastric adenocarcinoma epithelial cell line (AGS) cells	DN	DN	DN
L. fermentum	CRL-1831, Caco-2	DN	DN	ND	DN
L. salivarius	DN	MKN45 (gastric carcinoma) cells	DN	DN	DN
B. adolescentis	DN	DN	DN	DN	DN
L. rhamnosus	Human cell line (HGC-27), Caco-2 and HT-29	DN	DN	DN	DN
L. plantarum A7	Caco-2 and HT-29	DN	DN	DN	DN

Where DN indicates- Data not available

2.4.2.2 Animal model studies for anticancer activities

The anticancer effects of probiotics were determined by using the various animal models such as rats and mice. The positive results obtained gave the green signal to carry out the clinical trials on human volunteers. Many cancer inducer agents were used to develop cancer in the animals such as dimethylhydrazine (DMH), N-Methyl-N-nitrosourea (MMN) and azoxymethane (AOM) etc [92–97].

Many studies were recently carried out on *L. Acidophilus, L. Amylovorus, L. Brevis, L. Bulgaricus, L. Casei Immunitas, L. Casei, L. Crispatus, L. Delbrueckii, L. Fermentum, L. Gallinarum, L. Helveticus, L. Johnsonii, L. Johnsonii LC-1, L. Lactis, L. Plantarum, L. Reuteri, L. Rhamnosus, L. Salivarius, L. Sporogenes* for anticancer activity using the different animal models [97].

The different pharmacological effects of probiotics strains on animal models using the various chemical inducers for the cancer formations are as follows [59,96,98–103]

Table 2.11. Different probiotics used against the anti-cancer animal models

Sr. no.	Probiotics used	Cancer inducer	Animal
1	*L. acidophilus*	DMH	Sprague-Dawley rats, Wister rats
2	*B. fibrisolvens*	DMH	Male Jcl: ICR mice, F344 rats
3	*L. plantarum*	DMH	Wister rat, female Swiss albino mice
4	*L. fermentum*	DMH	Wister rat, female Swiss albino mice

5	*B. bifidum*	DMH	Wister rat, F344 rats
6	*L. casei*	AOM	Male C57BL/6J mice
7	*L. casei*	DMH	BALB/c mice
8	*L. rhamnosus*	DMH	Sprague Dawley rats, Wistar rats
9	*L. rhamnosus*	AOM	Male F344 rats
10	*L. helveticus.*	AOM	DBA/1J mice
11	*C. butyricum*	AOM	Male C57BL/6 mice
12	*B. lactis*	AOM	Wister rat
13	*L. paracasei*	MMN	Wister rat
14	*L. brevis*	MMN	Wister rat
15	*L. salivarius*	DMH	Male F344 rats

2.5 Clinical trials with different probiotics formulations

In clinical studies, probiotics are used against various intestinal disorders, including bacterial diarrhea, etc. Mostly, the probiotics are used as complimentary synergistic mediators during the chemotherapy and radiotherapy of the cancer patient [104]. *L. casei DN-114 001* was used during the radiotherapy and analyzed the progression in the host body by the stool consistency studies and bowel movements physiology [105]. Many researchers reported that the combination of *L. acidophilus* and *B. bifidum* minimize radiation-induced diarrhea [106]. Various therapies used

against cancers such as chemotherapy, changes the human gut microbiota inviting the pathogenic strains *Clostridium difficile* in the gut of the host observed in the patients, suffering from colorectal cancer [107,108]. The enteral administration of Bifidobacterium and Lactobacillus not only improved the patient's intestinal environments but also minimized the side effects of radiotherapy [109]. Many studies showed that the administration of the probiotics formulations reduced the post-operative trauma and intestinal infections [104,110]. Many probiotics decrease the chances of tumor formations induced by aflatoxin, used as a marker for liver cancer [111,112]. The current research showed that the inclusion of probiotics as a nutraceutical agent reduces the risk of breast cancer in women after menopause. The advantageous part is that consumption of these probiotics not affect the level of the hormone in these women during their reproductive phase of the life [28]. Probiotics were found effective against the atypia form of colorectal tumors in patients consuming the *L. casei* for a period of 4 years [72,110].

2.6 Conclusions

Probiotics and prebiotics are considered as vital contributor to functional food in the healing of gastrointestinal disorders. These microbes are used since a long time to treat many clinical disorders such as diarrhea and food allergies. Milk is the rich source of oligosaccharides. These carbohydrates act as the best prebiotics source for the growth of Lactobacillus. Lactobacillus was mostly discovered from the milk of different milking animals. These strains of Lactobacillus are nowadays analysed as a line of treatment against the various disease conditions of diabetes, cancer, obesity, and diseases of the gut. The major sources of these probiotics are from dairy and non-dairy fermented food products. Moreover, probiotics are screened for health benefits against intestinal pathogenic bacteria, lactose indigestions, an immunomodulator, allergic conditions, cardiac disorders, necrotising colitis and gingivitis. The mechanism to heal diseases by the probiotics are based on its various stress tolerance abilities like acid and bile tolerance, adhering to gut microvilli cells, and serviceability as supplements in various

diseased conditions. Therefore, the current research deals with the discovery of new strains and sub-strains of probiotics for their applications in biomedical and clinical research aimed to improve human health. Thus, the newly discovered probiotics are evaluated as the functional food found to treat various diseases. Thus, this science of nutraceuticals is emerging to find remedies against diseases such as cancer and gut disorders evaluated by animal studies and clinical trials. Thus, this review of probiotics and prebiotics will act as a landmark for the researchers working in the field of functional food and milk studies.

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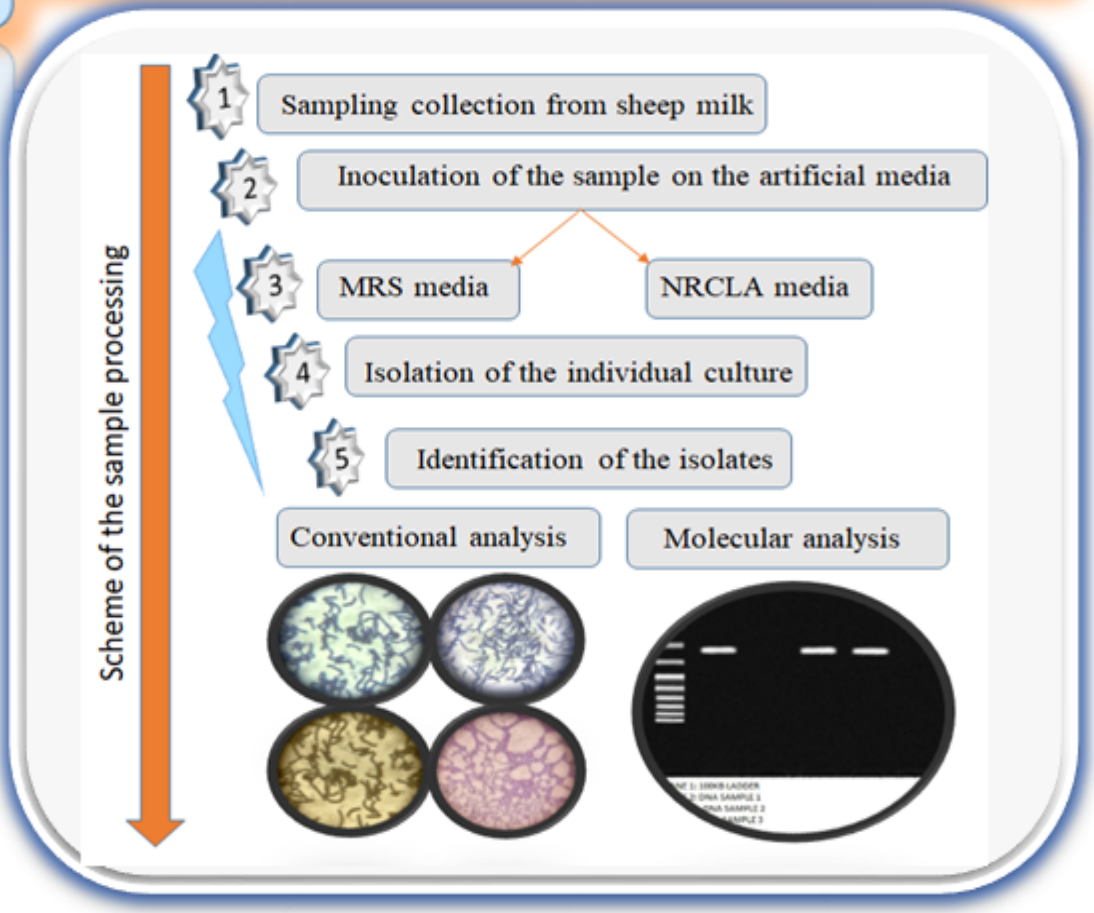
Chapter 3

Don't forget that the bacteria watch us from the other end of the microscope - Stanislaw J. Lec

Isolation and Identification of Lactobacillus from Sheep milk



3.1 Introduction

The current science of the nutraceutical world deals with the investigation of the new types of functional foods as an isolate for the betterment of the world [1]. The use of the microbes as functional food has explored its use in medical science. Probiotics are discovered from the different natural sources from time to time as the functional food. *Lactobacillus* is found as the most preferred genera in this direction with its utility in the dairy and allied sciences [2]. The most difficult task to use these microbes as the functional food is studying the growth parameters along with the genomic analysis. The phylogenetic relationship helps to identify the close resemblance of the discovered new strain of *Lactobacillus* with existing strains. The isolation of genomic DNA with amplification of 16 S rDNA region helps to identify the exact nature of the one strain with the others [3]. The common part observed in the case of probiotics from the same genera are its differences in physiological and biochemical characterizations [4]. Thus, to identify the exact difference between the closely related *Lactobacillus* from the same genera the whole genomic sequencing is carried out; this help to discover the special pharmacological importance of the discovered microbes belonging from the same family or the genera.

The present chapter deals with the isolation of the new strain of *Lactobacillus* along with the physiological, biochemical and genomic analysis of the same by various methods. In the current study, the two *Lactobacillus* strains are first time isolated from sheep milk; as earlier, it was reported to be found only in the human gut. Thus, these isolated cultures are comparatively studied with the existing culture of *L. acidophilus* obtained from NCIM (National Collection of Industrial Microorganism), Pune.

3.2 Experimental

Fig 3.1 represents the general plating method used for the isolation of lactic acid bacteria on artificial Lactobacillus selective media by taking sheep milk samples.

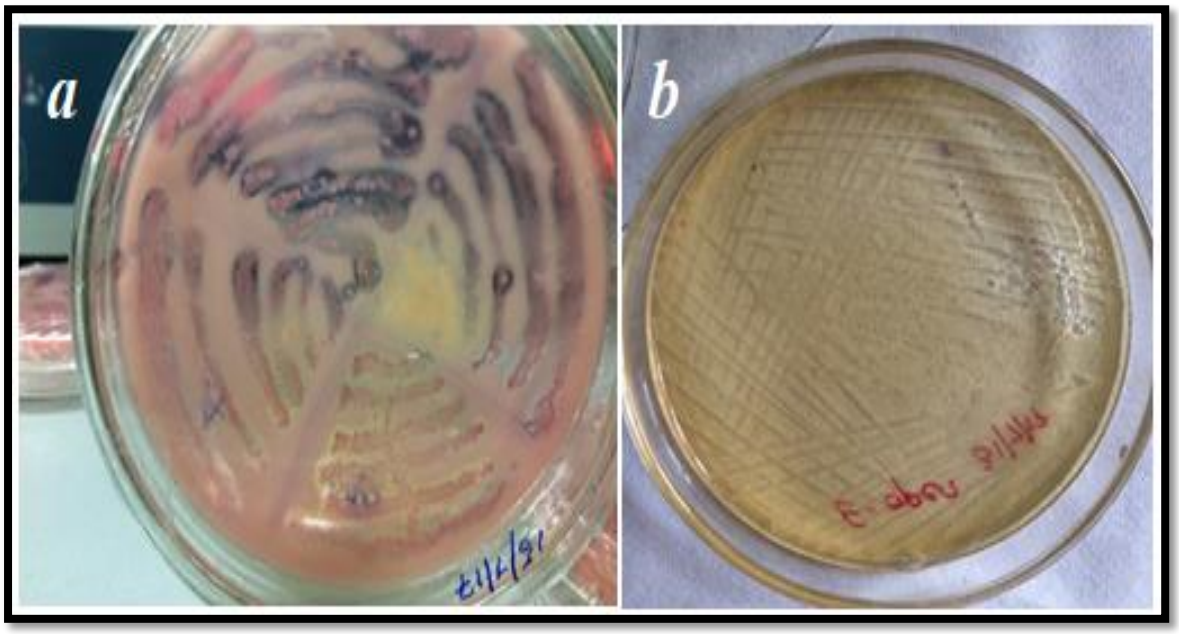


Fig.3.1. Isolation of the lactic acid bacteria on the a) NRCLA and b) MRS media from sheep milk samples

3.2.1 Isolation of lactic acid bacteria from sheep milk in selective media

180 milk samples were collected from the Indian sheep's breed from local places of Kolhapur, Sangli and Admapur areas of Maharashtra. The samples (50 ml) collected were stored at 5 °C until use. For bacterial enumeration, milk samples (1 ml) were kept at -78 °C in 15% glycerol before use. MRS (de Man, Rogosa & Sharpe) and NRCLA (Neutral Red Chalk Lactose Agar) broths and agar media Lactobacillus selective media were obtained as a gift sample from the Siffin Pharma, Germany [5]. MRS media was prepared by autoclaving 6.5 g MRS agar media in 100 ml distilled water, while NRCLA media was prepared by taking 5.1 g NRCLA agar media in 100 ml distilled water. The samples were inoculated on both MRS and NRCLA media by four quadrant streaking method and were incubated for a period of 48 h in a micro-aerophilic condition. After incubation, the individual colonies found on the NRCLA media were sub-cultured on MRS media and transferred into sterile MRS broth mediums. The purification of individually selected colonies were again carried out by the streak plate technique with the serial dilution method. The

isolated colonies were again kept at -78°C in 15% glycerol before use and were evaluated for their biochemical analysis.

Table 3.1. MRS media composition

Sr. no	Media ingredients	g/l
1	Proteose peptone	10.0
2	Beef extract	10.0
3	Yeast extract	5.0
4	Dextrose	20.0
5	Polysorbate 80	1.0
6	Ammonium citrate	2.0
7	Sodium acetate	5.0
8	Magnesium sulphate	0.1
9	Manganese sulphate	0.05
10	Dipotassium phosphate	2.0
Agar pH (25 ^{o}C) - 6.5 g/l (approximately)		
5.5 g/100ml distilled water at 15 lbs pressure (121 ^{o}C)		

Table 3.2. NRCLA media composition

Sr. No	Media ingredient	g/l
1	Peptic digest of animal tissue	3.0
2	Beef extract	3.0
3	Yeast extract	3.0
4	Lactose	10.0
5	Calcium carbonate	15.0
6	Neutral red	0.05
7	Agar	15.0
pH adjusted to 6.8 at 25°C		

Table 3.3. MRS broth composition

Sr. no	Media ingredients	g/l
1.	Peptone	10.0
2.	Lab-lemco powder	8.0
3.	Yeast extract	4.0
4.	Glucose	20.0
5.	Sorbitan mono-oleate	1.0 (ml)
6.	Tri-mmonium citrate	2.0
7.	Sodium acetate	5.0

8.	Magnesium sulphate	0.2
9.	Manganese sulphate	0.05
10.	Dipotassium phosphate	2.0
pH (25 °C) - 6.5 g/l (approximately)		
5.2 g/100ml distilled water at 15 lbs pressure (121 °C)		

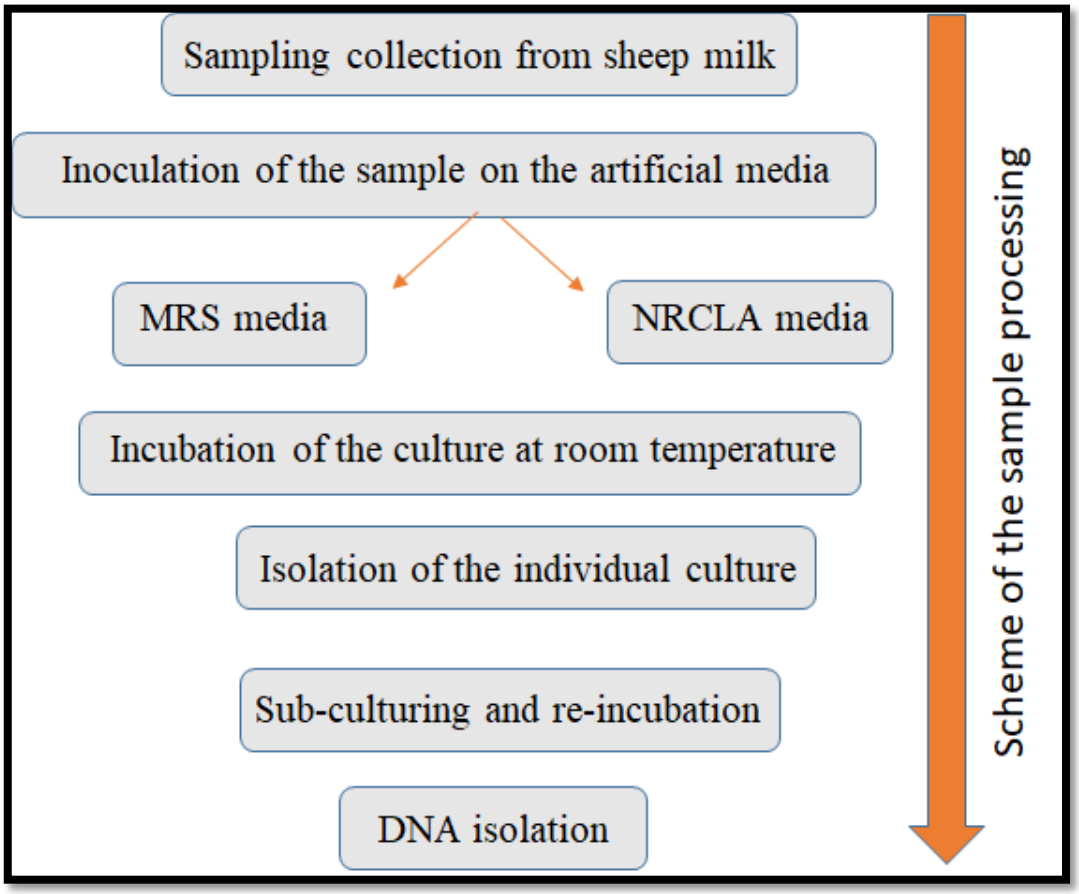


Fig. 3.2. Scheme of the microbial DNA sample processing

3.2.2 Conventional lab techniques for analysis of LAB

a) Gram Staining

The Gram staining of the isolates was determined by light microscopy using Gram staining reagents. It is known that LABs are gram-positive. This means that these cultures will produce blue-violet color for Gram-positive bacteria and vice-versa [6]. The cultures were grown in MRS media at 37 °C for 24 h under micro-aerophilic conditions. Fresh cultures were used for gram staining. After incubation, the cultures were aseptically transferred into 1.5 ml of eppendorf tubes and centrifuged for 3 min at 9000 rpm. The cells were resuspended in sterile water by removing the supernatant [5]. *L.acidophilus* from NCIM was used as positive control and *E. coli* was used as the negative control.

b) Catalase test

Catalase is an enzyme released by the microbes during the metabolic process. This enzyme act on hydrogen peroxide breaking it into water and oxygen and producing the gas bubbles. The release of the gas bubbles during the test indicates the presence of catalase enzyme.

$$2H_2O_2 \rightarrow 2\ H_2O + O_2 \qquad (3.1)$$

The catalase test was carried out on the isolates to see their reactions to catalase. To do this, two methods can be performed. 18 h incubated cultures of isolates were grown on MRS agar at room temperature. Furthermore, for the catalase test fresh liquid cultures of LAB were used in which 3% hydrogen peroxide solution was added to 1 ml of cultures [7].

c) Gas production from glucose

This test determines the hetero-fermentative and homo-fermentative nature of the isolates by the release of CO_2 production from glucose. The overnight 1% cultures of the isolates were inoculated in MRS broths lacking citrate into the

inverted Durham tubes. These cultures were further incubated for 48 h at 37 °C. The production of the CO_2 gas in Durham tubes indicates the presence of the glucose [5,8].

d) Growth at different temperatures

This test uses the bromecresol purple as an indicator in the freshly prepared MRS media. 50 µl overnight cultures of inoculum were added into 5 ml tube of modified MRS media and incubated for 7 days at 20 °C, 30 °C, 40, and 50 °C. During these incubation time, the change of the color from purple to yellow of the cells at different temperatures were observed [5,9]. *L.acidophilus* from NCIM was used as a positive control.

e) Arginine hydrolysis test

The arginine MRS modified medium and the Nessler reagent was used to view ammonia release from arginine. The freshly prepared 1% culture of the isolates was added into the MRS of 5 ml tubes containing 0.3% of L-arginine hydrochloride. The tubes were further incubated for 18 hours at 37 °C. After incubation, 50 µl of cultures were observed against the white background. 50 µl of the Nessler reagent was pipetted into the cultures and the change in the color was observed. The positive reaction was indicated by a bright orange color, while the yellow color determines the negative reaction. For the negative control, arginine free MRS was used [10].

3.2.3 Molecular analysis of Lactobacillus

3.2.3.1 Isolation of DNA from lactic acid bacteria

Milk of different animals were used to isolate the lactic acid bacteria. Frozen isolates were revived from the glycerol stock, thawed and re-inoculated into freshly prepared sterile MRS lactobacilli broth and incubated at 37 °C for 24 h [11]. After confirmation of purity, 10 µl of active broth culture was reinoculated into 10 ml sterile MRS broth and incubated at 37 °C for 10 h. Two-milliliter aliquots of active log phase cultures from this broth were then used to isolate genomic DNA.

The bacteria were collected by centrifugation at 5200 rpm for 5 minutes in a refrigerated centrifuge. The supernatant was decanted from the medium along with washing of pellet using 2 ml of NaCl thrice with EDTA (25 mM NaCl, 4 mM EDTA, at pH 8.0). Later, 100 ul freshly prepared lysozyme solution was mixed (concentration 10 mg/ml in NaCl-EDTA) and incubated at 37 °C for 60 min. 4 ul of RNase-A solution (10 mg/ml, working concentration 100 µg/ml) was used to remove the RNA before incubation. The final volume was made up to 500 microliters by addition of NaCl-EDTA, 12 ul of proteinase solution (17 mg/ml) and 50 ul of a 10% SDS solution. The contents were completely mixed and incubated at 50 °C for 55 min. After incubation, an equal volume of phenol saturated with Tris (pH 8.0) was mixed and centrifuged at 11,000 rpm at 21 °C for 600 s. The upper aqueous phase was carefully removed free from proteins and cellular residues. This phase was repeated once with a new aliquot of the phenol-chloroform mixture (1: 1) with a collection of the supernatant. The DNA in the supernatant was precipitated using 0.7 volumes of isopropanol along with 0.3 M sodium acetate (pH 5.5). The obtained DNA was pelletized by centrifugation at 9,000 rpm at 4 °C for 6 min. The obtained DNA in precipitated form was collected and dried by washing with a 71% ethanol solution. Furthermore, the granules formed were dissolved in 50 ul of Tris-EDTA (10: 1, pH 8) and stored at -18 ° C. In about three isolates were tested by using a commercial kit with the protocol suggested by the manufacturer. The DNA was stored frozen at -20 °C until use [12].

3.2.3.2 Amplification of 16S rDNA region, gel electrophoresis, and sequencing of LAB

A) Amplification of 16 S rDNA region of LAB

2 µl of genomic DNA was mixed with 48 µl of PCR mixture as per the instruction mentioned in the manufacturer user kit [13]. 50 µl of the final reaction mixture was taken to the PCR steps (Bio-Rad T 100 PCR, USA). The amplification was carried by using the EGE1 forward and EGE2 reverse primer of 16S rDNA region from the isolates. The forward primer is complementary to the 5'end of 16S

rDNA and the reverse primer is complementary to the 3' end of the 16S rDNA region.

Forward Primer: EGE1: 5'-AGGAGTTTATCCTGGCTCAG-3'

Reverse Primer: EGE2: 5'CTTACGGCACCTTGTTACGA-3'

The PCR Conditions:

Step 1: 94°C for 360 s

Step 2: 94°C for 60 s (denaturation)

Step 3: 56°C for 60 s (annealing)

Step 4: 72°C for 60 s (elongation)

Step 5: 72°C for 600 s

Table 3.4. PCR mixture composition

Sr. no	Media ingredient	Formula (µl)
1	Mg-freeTaq DNA polymerase buffer	5
2	$MgCl_2$ (25Mm)	3
3	Sterile deionized water	33
4	Oligo forward 10 picomole/µl	1
5	Oligo reverse 10 picomole/µl	1
6	dNTP (2 mM each)	5
7	DNA	2
Total		50

B) Separation of amplified PCR products

a) Preparation of agarose gel

0.8% prepared agarose gel was dissolved in boiling 100 ml TAE buffer. The gel was cooled at 45 °C with the addition of 15 µl ethidium bromide solution (9.9 µg/ml) was added. The agarose gel was placed into the gel casting stand along with the combs. The combs were removed after getting the rigid gel and used for loading the DNA samples [14].

b) Loading of Agarose Gel

2 µl loading dye was added to 5 µl of PCR products which was introduced into wells. The first well was loaded with the DNA size-marker (1 kb, Fermentas) to observe the amplification range.

c) Electrophoresis of the products

The electrophoreses of the PCR products were carried out at 79 mA for 40 min. The visualization of the PCR products was carried out by a gel documentation system (Vilber-Lormat). The amplication of the DNA fragments observed in the range 1500-2000 bp shows the proper output of the amplification.

d) Purification of PCR products

The restriction enzymes were used for PCR products prior to digestion. The purification procedure carried out were as follows:

• 50 µl 1XTE buffer was used to PCR product to make up the volume to 100 µl
• Addition and mixing of 200 µl chloroform solution was carried out in duplicate
• The formed solution was centrifuged at 5.000 rpm for 11 min
• The upper aqueous phase was mixed with the 0.l volume of 3 M sodium acetate (pH 5.2) solution
• Addition and mixing of 200 µl 99% ethanol solution was carried out in duplicate along with the centrifugation at 8.000 rpm for 10 min

• The supernatant was discarded and the formed DNA pellets were added with 500 µl of 70% ethanol

• The formed solution was centrifuged at 5.000 rpm for 11 min

• The formed pellet were dried at 37 °C for 10 min

• The formed DNA is dissolved into 50µl 1X TE solution

• DNA was stored as a solution at -20 °C.

e) DNA sequencing

The two isolates were sent out for 16S rRNA gene molecular identification, by illumination Nextseq platform, Netherlands. The results obtained after sequencing were then BLAST using the BLASTn algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi_) and identification of individual isolates were done. To elucidate the phylogenetic relation of our isolated *Lactobacillus*, we retrieved the 16S rRNA sequences of other *Lactobacillus* species from GenBank nucleotide database and the phylogenetic tree was constructed using the Maximum likelihood method.

C) Restriction fragment length polymorphism (RFLP)

11 µl of purified amplification PCR products were used for each of the restriction enzyme digestion. Three different enzymes *Bam HI*, *Hind* III, and *EcoR*I are used for enzyme digestion study. 50 µl final reaction volumes were used for digestion; for *Bam HI* at 65 °C, for *Hind* III and *EcoR*I, it was 37 °C. All of the reactions were performed overnight and additionally *Bam HI* restriction reactions were overlaid with mineral oil to avoid evaporation[15].

Table 3.5. RFLP reaction mixture composition

Sr. no	Media ingredient	Formula (µl)
1	Restriction enzyme buffer	5
2	Sterile deionized water	34.5
3	Restriction enzyme (5U)	0.5
4	DNA	10
	Total	50

a) Electrophoresis of restriction fragments

1.6% agarose gel was used for separation of restricted fragments.

b) Preparation of agarose gel

2.4 g agarose gel was dissolved in boiling 150 ml TAE buffer. The gel was cooled at 45 °C with the addition of 22 µl ethidium bromide solution (9.9 µg/ml) was added. The agarose gel was placed into the gel casting stand along with the combs. The combs were removed after getting the rigid gel and used for loading the DNA samples

c) Loading of agarose gel

In electrophoresis tank, the solidified agarose gel was placed with the pouring of 1.5 X TAE buffer containing 300 µl of ethidium bromide. 2 µl of gel loading dye was added to 11 µl solution of digestion products. The loading of the samples was carried out from the second well on the gel. 2 µl (500 ng) of DNA was loaded to the first well on the gel with the molecular weight marker from 100 bp to 1kb.

e) Electrophoresis of the products

The electrophoreses of the samples were carried out at 60 mA for 29 min and at 80 mA for 4 h. The visualization of the amplified products was carried out by a gel documentation system (Vilber-Lormat).

f) Interpretation of results

The images obtained by gel documentation were modified in Adobe Photoshop 7.0. using BIO-ID.++software (Vilbeer-L'ourmat). The strains similarities were observed automatically by using the formula of Jaccard. The unweighted_pair_group method was used for strain clustering studies using arithmetic averages and UPGMA, BIO-ID++ techniques. 10% of homology were prepared for the homology coefficient studies.

D) Whole genomic sequencing of Lactobacillus strains

The DNA libraries were generated as per manufacturers instruction using 'NextSeq-500/550 High-Output Kit version 02.5' (for 300/cycles) followed by sequencing carried out by NextSeq-500 sequencing_system-Illumina (The Netherlands). Trimmomatic v0.36 software was used for trimming of the reads applying sliding-window of 04:15 settings and minimum Phred score of 33. The reads were generated by removing the adaptor sequence. The read qualities were demonstrated by FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc). The assembling of data was carried out by de novo using SPAdes_v3.12.0. The further analysis was covered in the contigs >500 bp length and > 5x coverage.

3.3 Results and Discussion

3.3.1 Physiological and biochemical identification of LAB

All the isolates were subjected to Gram staining and they were examined under a light microscope (100X magnification). All the strains show blue-purple color staining, except *E. coli* which is used as a negative control reference. Hence

all the isolated strains are found Gram-positive bacteria (Fig. 3.3. A-C), while *E. coli* shows pink color as it is Gram-negative bacteria (Fig. 3.3.D).

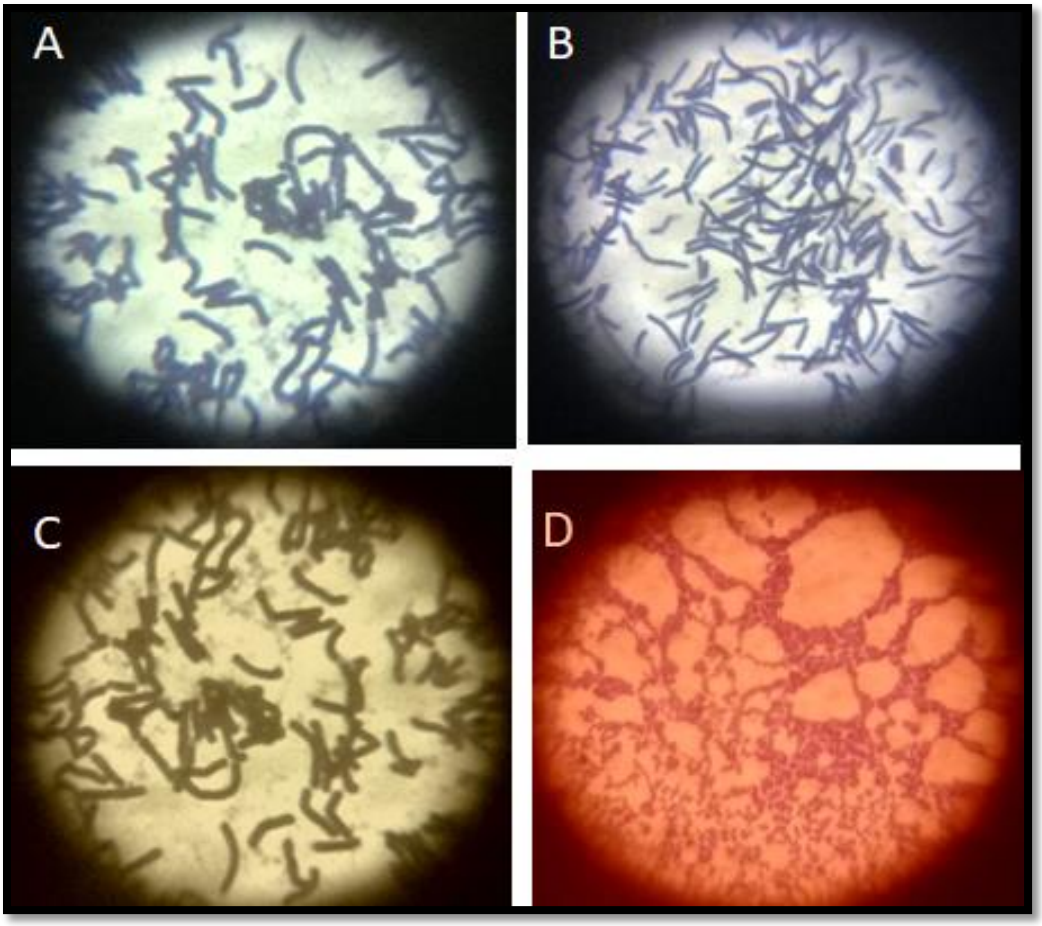


Fig. 3.3. Gram staining a) Sample A, b) Sample b, c) *L. acidophilus* (positive control) and d) *E. coli* (negative control) (100X)

The isolated Lactobacillus were long and rod-shaped. Isolates were tested for catalase activity. All isolates are catalase negative, as none of them given catalase activity. All strains show no gas production hence are homo-fermentative in nature (Fig. 3.4).

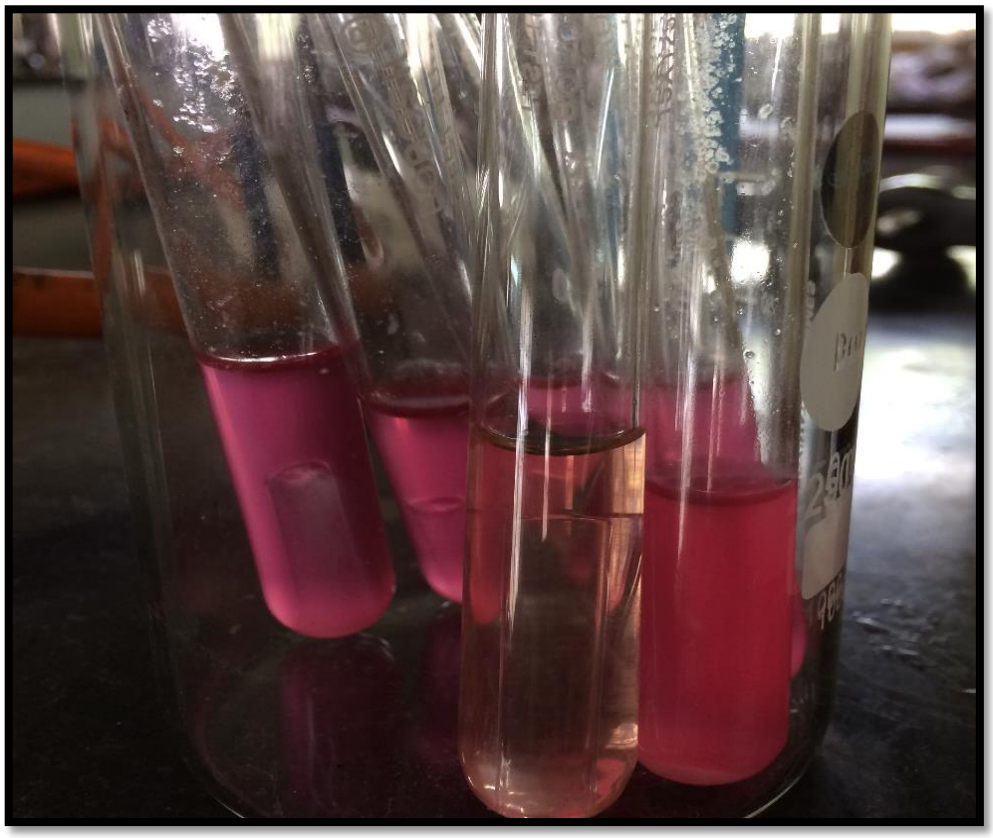


Fig. 3.4. Homofermentative nature observed in case of all Lactobacillus strain by Durham tube method

Another criterion for the identification of the isolates was the study of growth pattern at different temperatures. From the results of 7 days observation, all of the isolates show maximum growth between 35 °C ~ 37 °C. However, significantly very less growth is observed at 20 °C and 50 °C (Fig. 3.5).

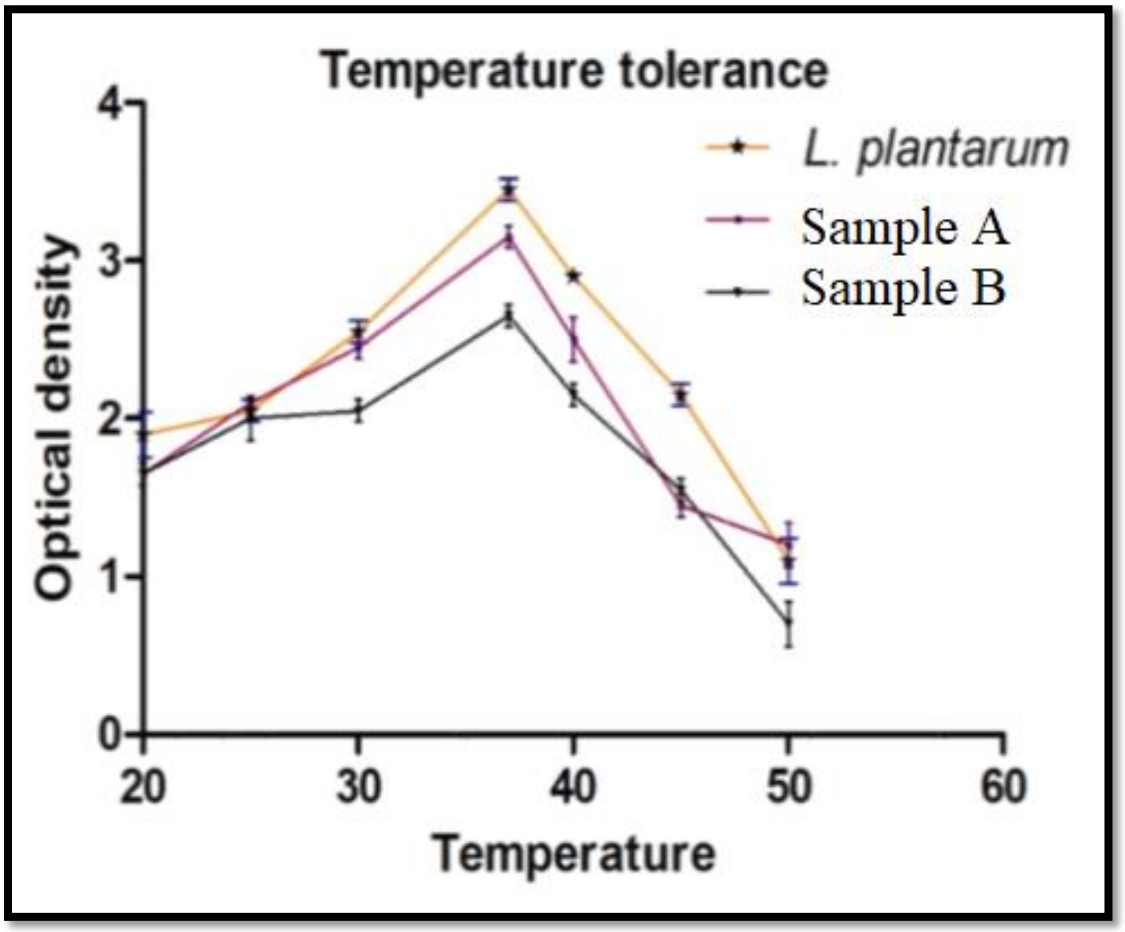


Fig. 3.5. Stress heat tolerance of Lactobacillus at various temperature

Arginine hydrolysis test was used as another step to follow the identification procedure. The isolates which gave the bright orange are found in producing ammonia from arginine. The yellow color indicated negative arginine hydrolysis. According to this test, both isolated strains produced ammonia from arginine.

3.3.2 Evaluation of amplification and digestion regions of 16S r DNA region of LAB

A) Molecular identification

a) Genomic DNA isolation

Genomic DNAs of the isolates are visualized by agarose gel electrophoresis under UV-light (Fig. 3.6). Then they are taken to the PCR step.

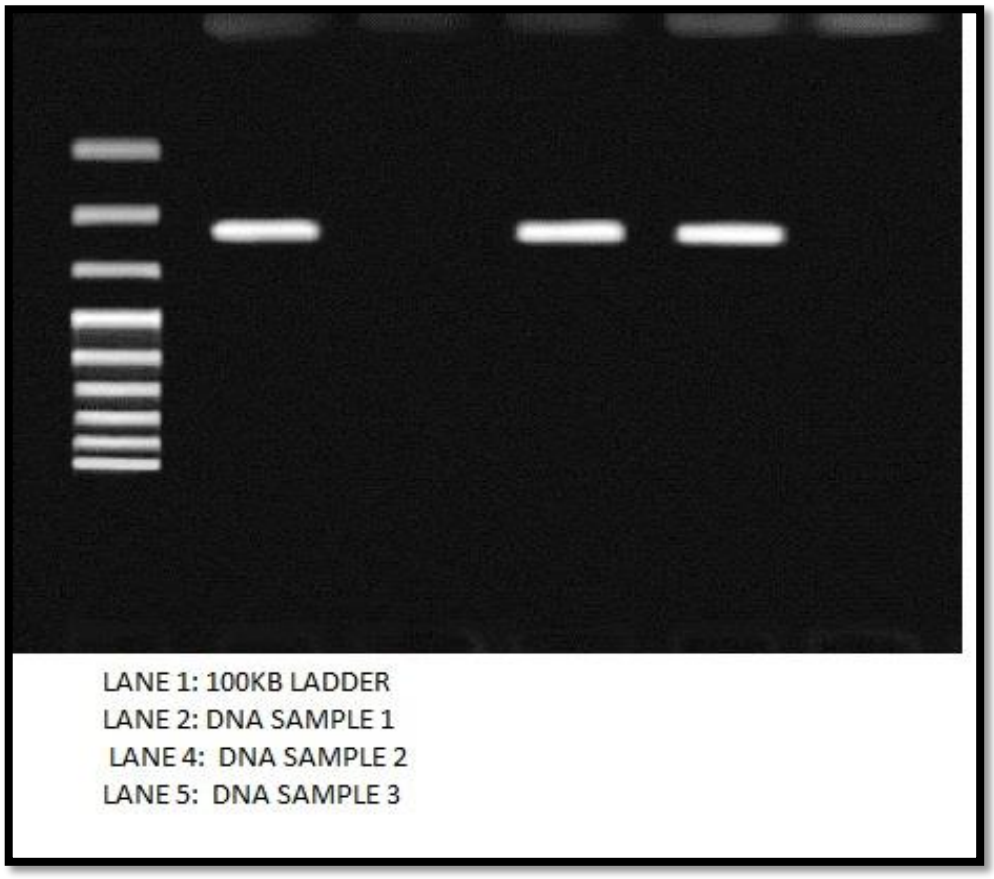


Fig. 3.6. DNA isolation Lactobacillus with the reference strain

b) Amplification of 16S rDNA region

After DNA isolation the 16S rDNA region is amplified by PCR protocol [3,10]. Then 50 µl of PCR products are visualized by agarose gel electrophoresis under UV-light. The length of the amplification products varied from 1850 to 2000 bp (Fig 3.7).

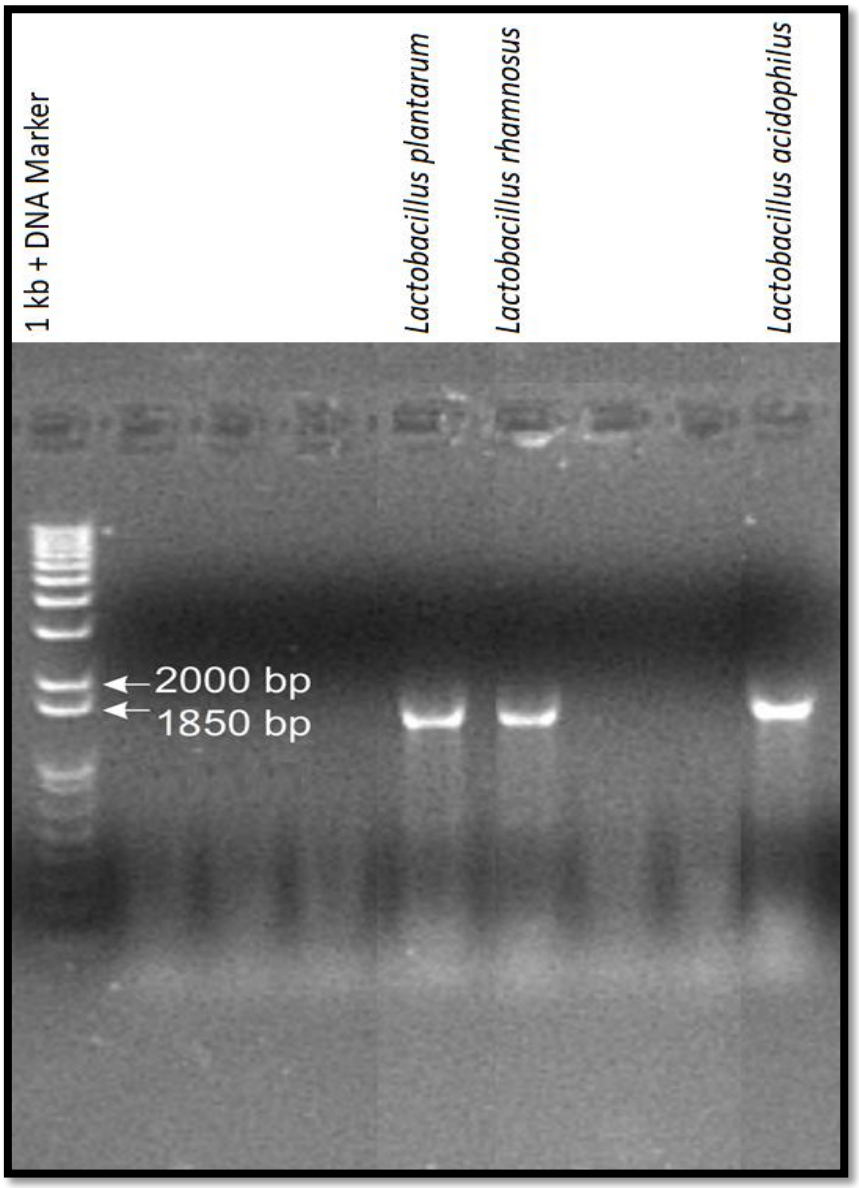


Fig 3.7. 16S Amplification products of isolates and reference strains

Identification of avian LAB isolates by 16S rRNA sequencing carried out by BLAST (Basic local alignment search tool) shows that sample A with 98% similarity with *Lactobacillus plantarum* (Lp). Similarly, sample B shows a 96% similarity with *Lactobacillus rhamnosus* (Lr). The correct identification of LAB with the accurate method and precision; having fast high discriminatory power is achieved by 16S rRNA gene sequencing [3].

Thus *L. plantarum* determined from (lane 5), *L. rhamnosus* (lane 6), and *L. acidophilus* (lane 9) used as the reference standard and are compared with 1kb DNA ladder gene ruler™.

c) Digestion of amplified 16S rDNA region by *Hind III, EcoR1, Bam H1*

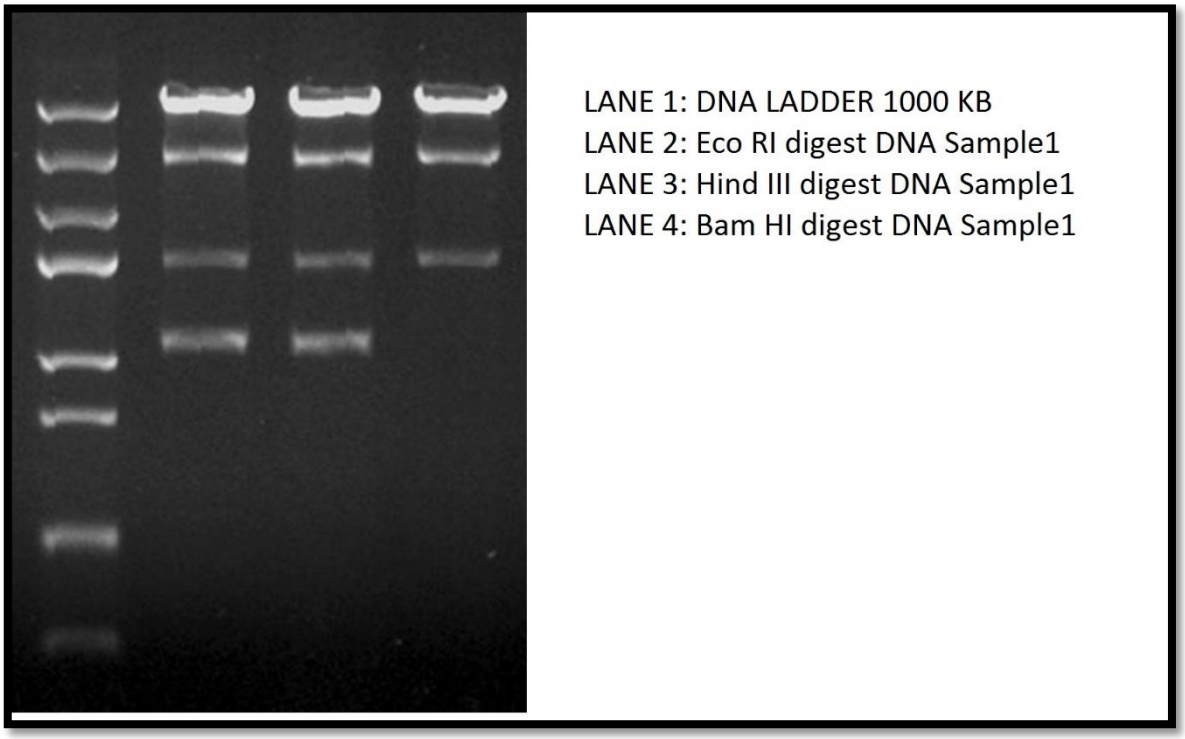


Fig 3.8. *Hind III, EcoR1, BAM H1* digests of *Lactobacillus plantarum*

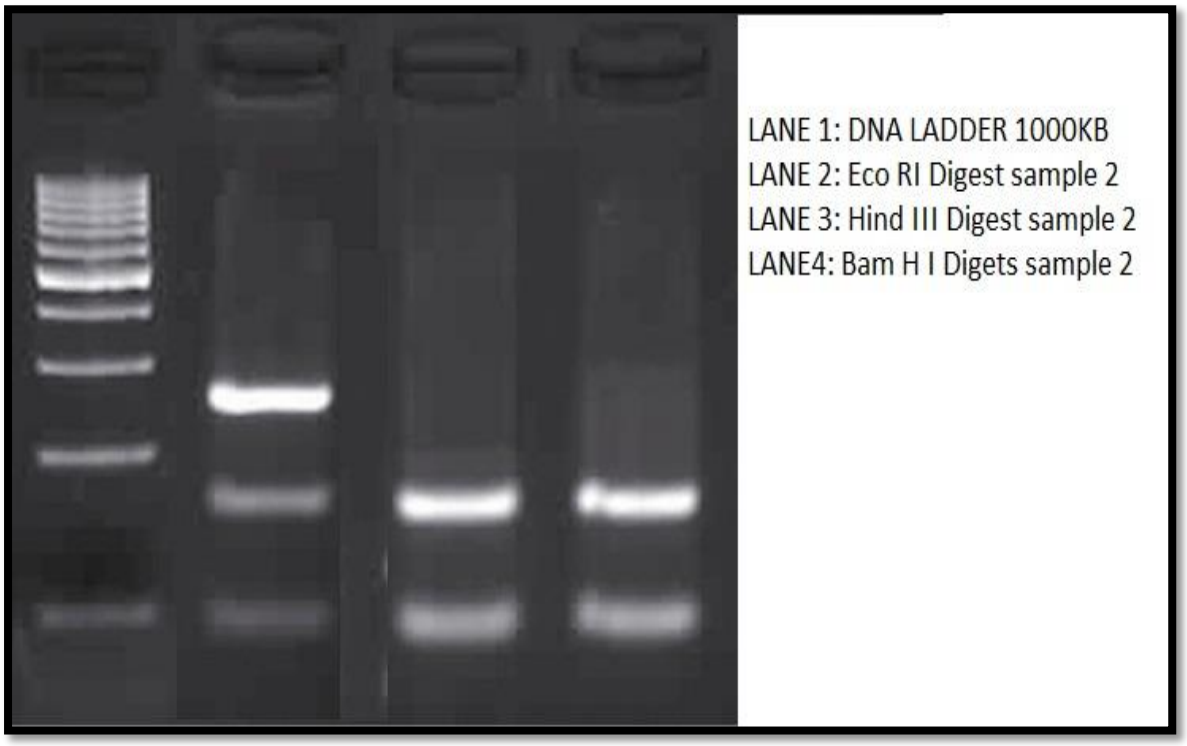


Fig 3.9. *Hind III, EcoR1, BAM H1*digests of *Lactobacillus rhamnosus*

The two isolates of *L. plantarum* (Fig 3.8) and *L. rhamnosus* (Fig 3.9) are digested by *Hind III, EcoR1, BAM H e*nzymes to get a restriction profile. It could be concluded that PCR-RFLP method by using *Hind III, EcoR1, BAM H1* enzymes revealed a good correlation between the two Lactobacillus strains. Because of the absence of some reference strains, isolates were identified by 16SDNA sequencing and BLAST in earlier studies [16].

3.3.3 Phylogeny identification and relationship of LAB

The phylogeny studies were carried out by the common ancestor's background determinations of the isolates. The Neighbor-Joining method is used in finding the evolutionary gaps and similarities [17]. The phylogeny tree with branch length sum is given as = 02.19005408 [17]. The tree is constructed with a scale, the same branch lengths are having the same units and determine the same evolutionary distances. The Maximum Composite Likelihood method is used in determining the evolutionary distances and are calculated as in the units with the proper substitutions

per locations. The analysis involves 24 nucleotide sequences (Fig. 3.10). The gap and missing links with its database are eliminated from the tree. 1011 positions data sets are finalized for the study. The study is conducted by using the evolutionary MEGA7 software.

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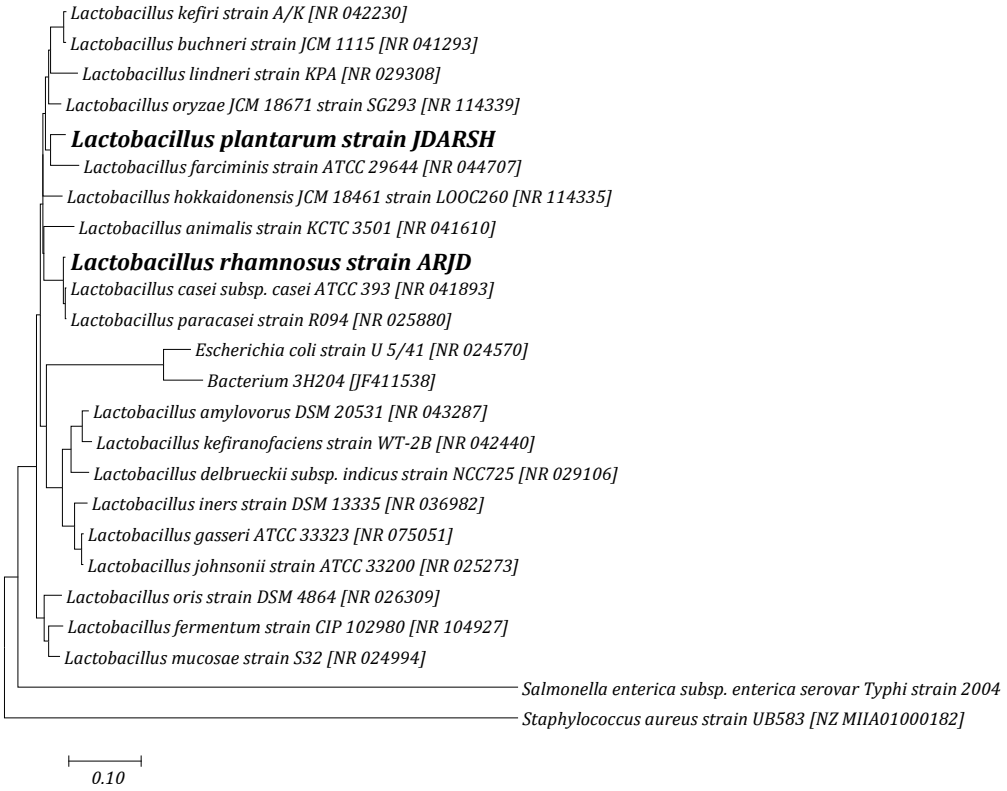

Fig 3.10. Evolutionary relationships of taxa by Neighbor-Joining method

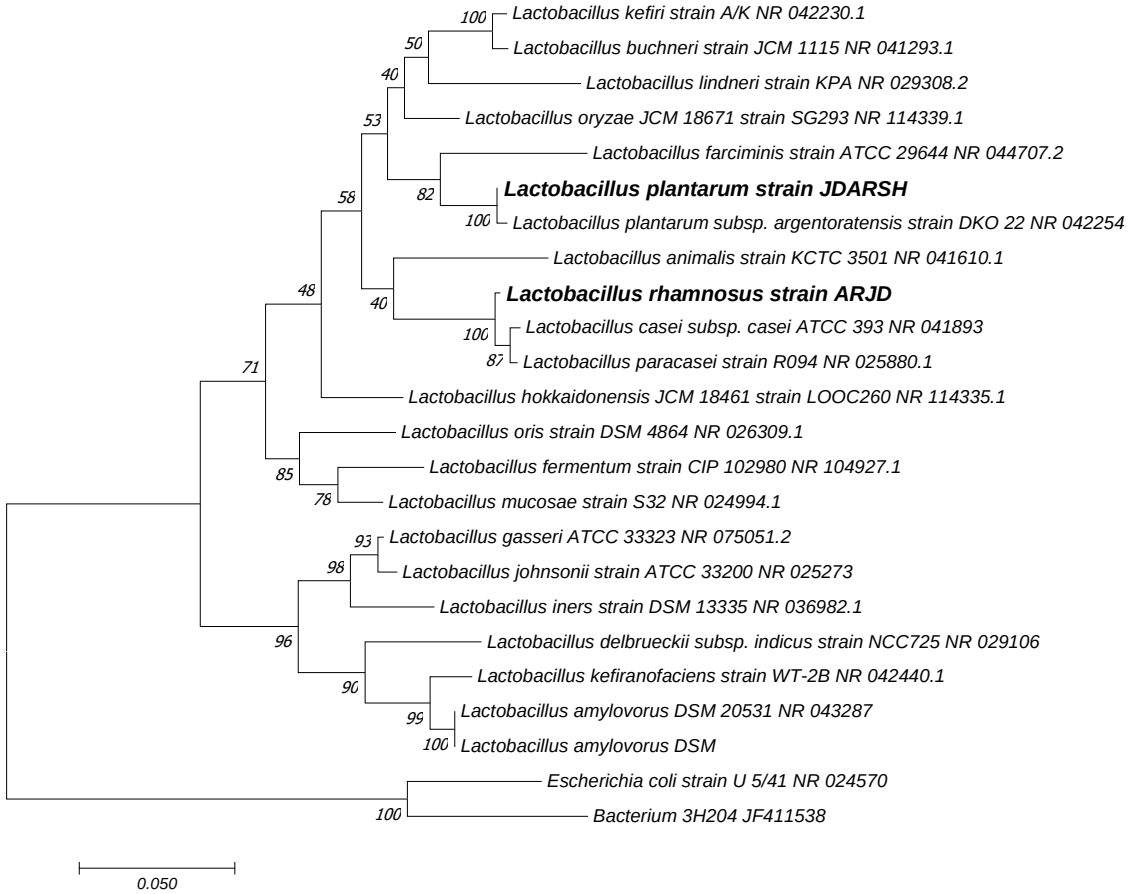

Fig 3.11. Evolutionary relationships of taxa by Maximum Likelihood method

Tamura-Nei model was used to study the evolutionary history by Maximum Likelihood method [18]. The chart prepared is a data sheet of the tree with the highest log likelihood (-07826.77) [19]. The similarity index in the form of the percentage i.e. associated similar taxas are clustered together and showed in next branches. The study was conducted using the Neighbor-Join and BioNJ algorithms studies plotting the initial tree(s) for the heuristic search using the Maximum Composite Likelihood (MCL) approach. The evaluation of the data is carried out by the topology superior log-likelihood value. The data sheet is presented as the tree with branch lengths with the substitutions at each branch. The analysis used 24 nucleotide sequences (Fig. 3.11). The gap and missing links with its database are eliminated from the tree. 1289 positions data sets are finalized for the study. The study is conducted by using the evolutionary MEGA7 software.

3.3.4 Whole genomic analysis of Lactobacillus strains (Lp, Lr)

a) Lactobacillus plantarum

LOCUS PYBS01000000: 31 rc DNA linear BCT 27-MAR-2018

DEFINITION *Lactobacillus plantarum* strain JDARSH, whole genome shotgun sequencing project.

ACCESSION PYBS00000000, VERSION, PYBS00000000.1

DBLINK Bio-Project: PRJNA439183; Bio-Sample: SAMN08741665

KEYWORDS-WGS.

SOURCE: *Lactobacillus plantarum*

ORGANISM: *Lactobacillus plantarum*

Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus.

REFERENCE-1: (bases 1 to 31)

AUTHORS: Patil, A., Suryavanshi, M., Disouza, J. and Pawar, S.

TITLE: Draft genome sequence of *Lactobacillus plantarum* strain JDARSH MCC3595

JOURNAL: Unpublished

REFERENCE: 2 (bases 1 to 31)

AUTHORS Patil, A., Suryavanshi, M., Disouza, J. and Pawar, S.

TITLE: Direct Submission

JOURNAL submitted (20-MAR-2018): Microbial Culture Collection, National Centre for Cell Science Pune, Pashan Sutarwadi, Pune, Maharashtra 411021, India

COMMENT: The *Lactobacillus plantarum* whole genome shotgun (WGS) project has the project accession PYBS00000000. This version of the project (01) has the accession number PYBS01000000, and consists of sequences PYBS01000001-PYBS01000031.

Bacteria and source DNA available from MCC 3595.

Annotation was added by the NCBI Prokaryotic Genome Annotation

Pipeline (released 2013). Information about the Pipeline can be found here: https://www.ncbi.nlm.nih.gov/genome/annotation_prok/

##Genome-Assembly-Data-START##

Assembly Date: MAR-2018

Assembly Method: SPAdes v. 3.9

Genome Representation: Full

Expected Final Version: Yes

Genome Coverage: 102.0x

Sequencing Technology: Illumina HiSeq

##Genome-Assembly-Data-END##

##Genome-Annotation-Data-START##

Annotation Provider: NCBI

Annotation Date: 03/20/2018 12:37:21

Annotation Pipeline: NCBI Prokaryotic Genome

Annotation Pipeline

Annotation Method: Best-placed reference protein set; Gene MarkS+

Annotation Software revision: 4.4

Features Annotated: Gene; CDS; rRNA; tRNA; ncRNA; repeat_region

Genes (total): 3,128

CDS (total): 3,060

Genes (coding): 2,980

CDS (coding): 2,980

Genes (RNA): 68

rRNAs: 3, 1, 1 (5S, 16S, 23S)

Completer RNAs: 1, 1 (16S, 23S)

Partial rRNAs: 3 (5S)

tRNAs: 59

ncRNAs: 4

Pseudo Genes (total): 80

Pseudo Genes (ambiguous residues): 0 of 80

Pseudo Genes (frame shifted): 39 of 80

Pseudo Genes (incomplete): 27 of 80

Pseudo Genes (internal stop): 24 of 80

Pseudo Genes (multiple problems): 10 of 80

CRISPR Arrays: 1

##Genome-Annotation-Data-END##

FEATURES- Location/Qualifiers source: 1….31

/organism= "Lactobacillus plantarum"

/mol_type= "genomic DNA"

/strain= "JDARSH"

/isolation_source= "milk sample"

/culture_collection= "MCC: 3595"

/db_xref="taxon: 1590" /country="India: Kolhapur" /collection_date= "2016"

/collected_by= "Mr. Abhinandan Patil"

WGS: PYBS01000001-PYBS01000031

Thus, *Lactobacillus plantarum*is designated as sample A (Lp) for the further experimental processes.

b) Lactobacillus rhamnosus

LOCUS PYRN01000000, 38 rc DNA; linear- BCT 18-SEP-2018

DEFINITION: *Lactobacillus rhamnosus* strain ARJD, whole genome shotgun sequencing project.

ACCESSION: PYRN00000000

VERSION: PYRN00000000.1

DBLINK: Bio-Project: PRJNA445595; Bio-Sample: SAMN08793894

KEYWORDS- WGS

SOURCE: *Lactobacillus rhamnosus*

ORGANISM: *Lactobacillus rhamnosus*

Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus.

REFERENCE: 1 (bases 1 to 38)

AUTHORS: Patil, A., Suryavanshi, M., Disouza, J. and Pawar, S.

TITLE: Draft genome sequence of *Lactobacillus rhamnosus* strain ARJD MCC 3594

JOURNAL- Unpublished

REFERENCE- 2 (bases 1 to 38)

AUTHORS-Patil, A., Suryavanshi, M., Disouza, J. and Pawar, S.

TITLE-Direct Submission

Journal Submitted (25-MAR-2018) Microbial Culture Collection, National Centre for Cell Science Pune, Pashan Sutarwadi, Pune, Maharashtra 411021, India

COMMENT: The *Lactobacillus rhamnosus* whole genome shotgun (WGS) project has the project accession PYRN00000000. This version of the project (01) has the accession number PYRN01000000, and consists of sequences PYRN01000001-PYRN01000038.

Bacteria and source DNA available from MCC 3594.

Annotation was added by the NCBI Prokaryotic Genome Annotation

Pipeline (released 2013). Information about the Pipeline can be found here: https://www.ncbi.nlm.nih.gov/genome/annotation_prok/

##Genome-Assembly-Data-START##

Assembly Date: MAR-2018

Assembly Method: SPAdes v. 3.9

Genome Representation: Full

Expected Final Version: Yes

Genome Coverage: 110.0x

Sequencing Technology: Illumina HiSeq

##Genome-Assembly-Data-END##

##Genome-Annotation-Data-START##

Annotation Provider: NCBI

Annotation Date: 03/26/2018 23:45:36

Annotation Pipeline: NCBI Prokaryotic Genome

Annotation Pipeline

Annotation Method: Best-placed reference protein set; Gene MarkS+

Annotation Software revision: 4.4

Features Annotated: Gene; CDS; rRNA; tRNA; ncRNA; repeat_region

Genes (total): 2,937

CDS (total): 2,872

Genes (coding): 2,773

CDS (coding): 2,773

Genes (RNA): 65

rRNAs: 1, 1, 1 (5S, 16S, 23S)

Completer RNAs: 1, 1, 1 (5S, 16S, 23S)

tRNAs: 59

ncRNAs: 3

Pseudo Genes (total): 99

Pseudo Genes (ambiguous residues): 0 of 99

Pseudo Genes (frame shifted): 45 of 99

Pseudo Genes (incomplete): 39 of 99

Pseudo Genes (internal stop): 29 of 99

Pseudo Genes (multiple problems): 13 of 99

CRISPR Arrays: 1

##Genome-Annotation-Data-END##

FEATURES: Location/Qualifiers source-1.38

/organism= "*Lactobacillus rhamnosus*"

/mol_type= "genomic DNA"

/strain= "ARJD"

/isolation_source = "milk"

culture_collection = "MCC: 3594"

/db_xref="taxon: 47715" /country="India: Kolhapur" /collection_date="2016"

/collected_by= "Mr. Abhinandan Patil"

WGS: PYRN01000001-PYRN01000038

Thus, *Lactobacillus rhamnosus* is designated as sample B (Lr) for the further experimental processes.

3.4. Conclusions

The present topic focuses on the isolation of the new strains of Lactobacillus i.e. *L. plantarum* (Lp) and *L. rhamnosus* (Lr) from the sheep milk. Based on the positive results of the morphological investigation, the DNA of the strains are isolated and analysed for the 16 S rDNA investigation by taking standard reference strain of *L. acidophilus* (La). On the basis of the difference in the biochemical investigation, the whole genome sequence analysis is carried out and culture are deposited as *L. plantarum* MCC 3595 and *L. rhamnosus* MCC 9594 in NCIM, Pune.

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Chapter 4

The part of this chapter have been published as a research article

In-vitro gastrointestinal simulated studies of Lp, Lr and La as Lactobacillus

Discovery of new strains of Lactobacillus

Culturing and sub-culturing of the strains to evaluate the efficiency of the same

Study of the probiotic properties

A) Organic acid and pH value estimation
B) Sugar fermentation test
C) Bile tolerance activity
D) NaCl tolerance
E) Resistance to phenol
F) Cholesterol assimilation
G) Lysozyme resistance
H) Negative hemolytic and Dnase activity etc

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RESEARCH ARTICLE

Probiotic Potential of *Lactobacillus Plantarum* with the Cell Adhesion Properties

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Shelf life stability of encapsulated lactic acid bacteria isolated from sheep milk thrived in different milk as natural media

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4.1 Introduction

The Lactobacillus genera from the probiotics category are found in the milk of different animals. These microbes are used since a long period as a functional food to treat many gastrointestinal related disorders. LAB's is the most acceptable form of probiotics, obtained mostly from milk and milk products [1]. These microbes act as a nutraceutical agent conferring health benefits. The LAB's such as *Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus plantarum,* etc., are being used by many dairy industries as the active starter cultures for many preparations including milk products and beverages [2]. Many researchers use fruit juices as natural media to enhance the growth and survival of these bacteria. The various properties of Lactobacillus i.e. its survival in the gastrointestinal tract is very important aspects [3]. Various stress boosters are present in the gut region of the host that interact with the microbial flora. These stress conditions include the effects of different enzyme and enzymatic system [4]. The major other factors affecting the growth and survival of the LAB are the bile salt secretion and the effects of the cellular immunity system inside the gut of the host. The phenols are the compound secreted by the pathogenic strains inside the gut lumen which may inhibit the growth of LAB's. The resistant ability of the LAB against the phenol not only greatly affect the survival of Lactobacillus against the dysbiosis condition but also helps in maintaining the microbiota of the same. Similarly, the stomach duodenum passage is the area of higher lysozyme and bile salt hydrolase activity [5]. Any probiotics by-passing these stress conditions and showing the tolerance against it are real contributors in showing the desired pharmacological activities.

Many synthetic moieties used in the processing of the food is found exhibiting toxicity by means of the haemolytic and DNase activity [6]. So functional foods like Lactobacillus should be evaluated for these types of toxicity [7]. Many synthetic formulations are available in the market showing cholesterol-reducing abilities. The heavy and consistence consumptions of these types of drugs precipitate cellular or hepatotoxicity [8]. Currently, many functional food i.e. probiotics are

coming in the market that greatly showing cholesterol assimilation and other probiotics properties which come with nearly zero adverse effects. The drawbacks of currently available nutraceutical foods available in the market come with the one or two pharmacological applications [6]. On the contrary, the pharmaceutical industries especially the nutraceutical world are looking for new types of formulation exhibiting all functional abilities in the single formulation.

In the current study, the activities of the LAB (Lp, Lr, and La) against various gut stress conditions are analysed. The *in-vitro* gastrointestinal simulated studies are carried out to explore the abilities of LAB to withstand gastrointestinal stress conditions.

4.2 Experimental

Fig 4.1 represents the general methodology used for the study of the LAB for its various stress tolerance conditions, especially based on its simulated habitat (gastrointestinal tract).

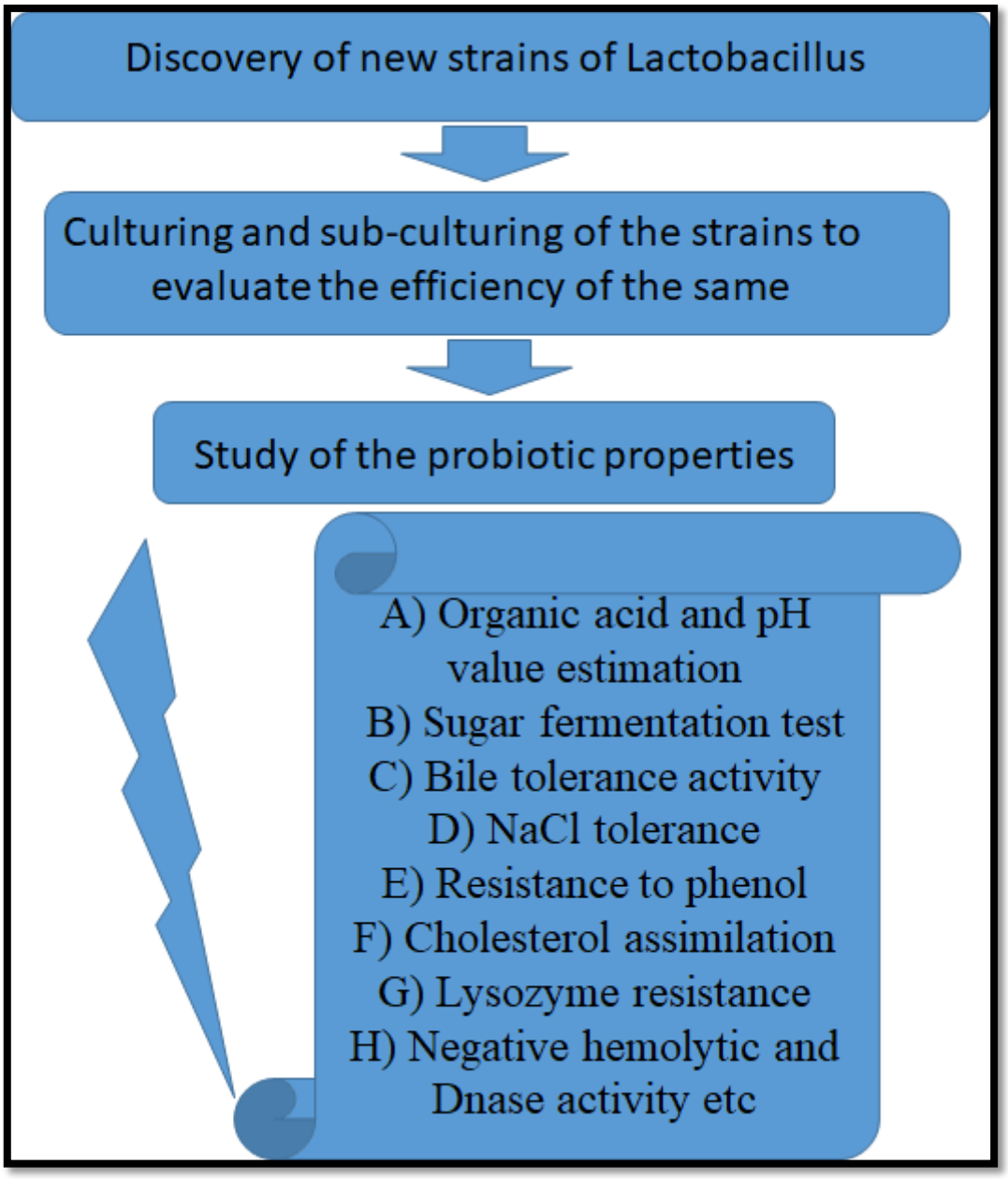

Fig. 4.1. Schematic pathway of culturing of Lactobacillus with its study of simulated gastrointestinal stress conditions

4.2.1 Investigation of LAB resistance to simulated gastrointestinal conditions

4.2.1.1 Sugar fermentation test

The sugar fermentation test was carried out using the pH indicator method according to the procedure described by Goyal et al. [9] with slight modification. The

test was performed using phenol red (0.01 g) as a pH indicator added in MRS broth at pH 6.5 in a test tube. The medium was autoclaved at 121 °C for 20 min by addition of 1 ml filtered and sterilized different sugar solutions such as ribulose, sorbitol, sucrose, fructose, cellobiose, salicin, lactose, and mannitol. Then 250 µl of an overnight bacterial culture was inoculated into the broth medium and incubated anaerobically at 37 °C for 24 h. The acid production in broth changed the color of medium to yellow. Later, Durham tubes were introduced into each culture tube in order to observe gas production.

4.2.1.2 NaCl tolerance test

NaCl tolerance test was performed according to the method described by Menconi et al. [10] with slight modification. It included 10 test tube containing MRS broth which was adjusted with different concentration of NaCl (1-10%). Each test tubes after sterilization were inoculated with 1% (v/v) fresh overnight cultures of the LAB; further incubated at 37 °C for 24 h. After 24 h of incubation, their growth was determined by observing the culture medium's turbidity. At the time points of evaluation, each sample in series from 1% (v/v) to 10% (v/v) were streaked onto MRS agar media to determine tolerance and viability of the strains in cfu. Each experiment were performed in triplicate n=3*.

4.2.2 Estimation of organic acid and pH value of Lactobacillus

The determination of the percentage of organic acid and pH values of LAB was carried out according to the method described by Olivera et al. [11] with the slight modification. Active cultures of the selected LAB (1%) obtained from 24 h broth culture were inoculated in 10% diluted sterilized buffalo milk at initial pH (6.71), which were measured by a DBK labline digital electrode pH meter. The inoculated milk was further incubated at 37 °C for 24 h, 48 h, and 72 h and samples were collected at each interval. Samples were filtrated aseptic and analyzed by performing titration with 0.1 N NaOH. The pH of the separated liquid was recorded using a digital electrode pH meter.

4.2.3 Studies on the response of LAB against *in-vitro* physiological stress conditions

4.2.3.1 Bile tolerance activity

Bile tolerance test was performed as per the procedure described by the Hassanzadazar et al., [12] (2012) with the slight modification. To evaluate the survival of selected LAB in the presence of bile salts, aliquots of active cultures grown in MRS broth for 24 h at 37 ºC were adjusted to pH 4.5 with sterile 1 N HCl or 1 N NaOH. Concentrated bile solution was prepared separately by dissolving powdered bile extract which was then sterilized and filtered by the 5-micron filter paper to achieve a final concentration of 1.0% and 1.5% and the third 0.0% culture served as a control sample. The culture was incubated at 37 ºC for 3 h to give the simulated condition of the intestine as the food has the transit period of 3 h in it. Samples were taken every hour after adding the bile solution from the period of 0 h to 3 h respectively. 10-fold serial dilution pour plate method was employed of all the samples by using 0.1% peptone water. The experimental analysis was carried out in triplicate.

4.2.3.2 Cholesterol assimilation test by Lactobacillus

This assimilation test was carried out on the LAB strains with slight modifications. The microbes were inoculated with the solution of acid cholesterol with the final volume of 100 mg/l and oxgall with a concentration of 0.2% or 0.4% w/v into MRS broth of 10 ml. These final solutions were incubated at 37 °C for 12 h under microaerophilic conditions. Further, the centrifugation (2700 g for 8 min at 4 °C) was carried out to remove the cells, the separated amount of supernatant was measured. The separated cells after drying at 75 °C in the oven were resuspended in sterile milli-Q water. The optical density of the samples was measured in aliquots every hour at 640 nm in which plain MRS broth was treated as control. The cholesterol assimilation percentage was estimated by the equation 4.1 [5].

$$CH = BC \times 100 \quad (4.1)$$

Where CH is the cholesterol of the separated pellet, B is the sample absorbance with the cells, and C is sample absorbance without the cells. The samples without cells contained no pellet, still, the cholesterol level was calculated in both the system.

4.2.3.3 Resistance to phenol

Few of the gut microbes are with the ability to produce the phenols by deaminating the aromatic amino acids from dietary proteins. The LAB mostly get inhibited by the phenolic compounds. The probiotics showing resistance to the phenolic compound shows the higher survival rate in the alimentary-canal of the host body [5]. The LAB cultures were developed in the MRS broth at 24 h incubation for overnight. This culture is incubated with 0.4% phenol for a further 24 h at 37 °C. Further, by the serial dilution method, the cultures were plated on the MRS media and cell count was determined.

4.2.3.4 Lysozyme resistance

The Lactobacillus isolates cultures developed in the MRS broth at 24 h incubation for overnight were centrifuged (6500 rpm, 8 min, 4 °C). The pellets of cells were formed by washing with the PBS solution. These are inoculated in the Ringer solution. 10 µl of the formed solution was suspended into the 100 mg/l lysozyme solution along with the electrolyte solution of KCl 1.9 g/l, NaCl 5.9 g/l, $CaCl_2$ 0.21 g/l and $NaHCO_3$ 0.9 g/l). The isolates without the lysozyme and electrolyte were treated as control the group for the analysis. The plate count was carried out by incubating the cultures at 37 °C for 2 h [5].

4.2.3.5 Bile salt hydrolase activity (BSH)

This assay was performed on the selected strains of the isolates as per Yadav et al. [5]. The Lactobacillus isolates cultures developed in the MRS broth at 24 h were incubation for overnight. These cultures were streaked on modified MRS medium enriched by 0.29 g/l $CaCl_2$ and 0.4% (w/v) bile salts. Further, the plates were

incubated for 48 h at 37 °C. The formation of the hydrolysed precipitation zones by salt activity nearby colonies determines the BSH activity. The studies were carried out against the three different bile salt i.e. sodium taurodeoxycholate (TDC), sodium tauroglycocholate (TGC) and sodium taurocholate (TC).

4.2.3.6 Response to stomach-duodenum passage (SSDP) simulation study

It is a very important assay which determines the survival of the LAB in gastrointestinal tract especially in the stomach-duodenum region [5]. For this, acidified MRS broth (pH 2.9) was developed and enriched with the simulated duodenum juice containing KCl 0.19 g, NaCl 1.31 g, Oxgall solution (10 g/100 ml) and Na_2HCO_3 6.4 g in 1000 ml distilled water for 20 h at 37 °C. Further, the culture was redeveloped in the MRS broth for 37 °C. The viable count was determined by taking the aliquots of the sample after 0, 1 h by the spread plate technique. Further, the samples were re-incubation for 60 min with 18 ml duodenum juice and 4 ml oxgall. The viable count was determined by taking the samples after 2.5 h and the percent survival rate was calculated by plating.

4.2.3.7 Hemolytic activity

The LAB cultures developed in the MRS broth at 24 h incubation for overnight were streaked on agar plates enriched by the blood (7% v/v slaughterhouse goat's blood). Further, the plates were incubated for 48 h at 37 °C and zone formation of the hemolysed patches around colonies was observed [5].

4.2.3.8 DNase activity

The LAB cultures developed in the MRS broth at 24 h incubation for overnight were streaked on DNase agar medium to evaluate the formation of the DNase enzyme. Further, the plates incubated for 48 h at 37 °C were observed for DNase activity in the form of the clear pinkish zone around Lactobacillus colonies [5].

4.3 Statistical analysis

One way analysis of variance (ANOVA) with Tukey's multiple comparison tests was used to compare the results of the probiotic and control groups using GraphPad Prism 5.01 software, USA. Standard deviations and significant differences were presented as $*p<0.05$, $**p<0.01$.

4.4 Results and Discussion

4.4.1 Evaluation of LAB resistance to simulated gastrointestinal conditions

4.4.1.1 Sugar fermentation pattern

Isolated bacteria showed a different pattern of sugar fermentation tests as recommended by Harrigan et al., [13]. Lp, Lr, and La were selected and subjected to a sugar fermentation test at a selected optimised temperature of 37 ^{o}C. In this test, mainly fermentation pattern of different sugars by LAB and no gas production are observed (Table 4.1). The isolated strain Lp shows maximum sugar fermentation pattern as compared to strain La which is showing minimum value.

Table 4.1. Sugar fermentation test of isolated LAB grown in buffalo milk

Name of the bacterial isolate	Rib	Sor	Man	Suc	Frt	Cel	Sal	Lat	Gas production
Lp	+	+	-	+	+	+	+	+	No
Lr	+		-	-	+	-	-	+	No
La	+	+	-	+	+	+	+	+	No

Rib- Ribulose, Sor- Sorbitol, Suc=Sucrose, Frt=Fructose, Cell=Cellobiose, Sal=Salicin, Lact=Lactose, Man- Mannitol (+) means good fermentation and acid production, (-) means no fermentation and no acid production. Lp, Lr, and La are

the (*L. plantarum, L. rhamnosus, and L. acidophilus*) LAB isolated from sheep milk but are grown in the milk of buffalo.

4.4.1.2 Tolerance to NaCl

The identified LAB from sheep milk is able to tolerate 1-10% NaCl. The results are presented in Table 4.3. The study shows a decline in the growth rate as from 10^{10} cfu/ml to 10^{4} cfu/ml by a gradual increase in the concentration of NaCl concentration. The result observed in case of all LAB strains appeared similar as compared to each other. NaCl concentration above 8% demonstrated as fatal to LAB showing no growth in MRS agar by serial dilution plate count method. NaCl tolerance test shows that at highest stress concentration of NaCl i.e. 7% v/v Lp is with a good survival rate of about 'X x $10^{4'}$ cfu/ml (Table 4.2).

Table 4.2. Effect of NaCl concentration on LAB isolates grown in buffalo milk

Concentration of NaCl (%)	Isolate Sample (cfu/ml) *		
	Lp*	Lr*	La*
1	~ 11 ± 7.2▲	~ 10 ± 2.2▲	~ 12 ± 7.5▲
2	~ 21 ± 5.2 ●	~ 24 ± 8.5 ●	~ 23 ± 5.8 ●
3	~ 12 ± 8.4 ●	~ 11 ± 6.4 ●	~ 12 ± 8.4 ●
4	~ 11 ± 4.1 ■	~ 15 ± 4.9 ■	~ 11 ± 4.6 ■
5	~ 16 ± 9.9 □	~ 19 ± 6.5 □	~ 16 ± 9.1□
6	~ 14 ± 8.5 ○	~ 19 ± 6.4 ○	~ 04 ± 5.5 ○
7	~ 09 ± 2.3 ◊	~ 08 ± 2.9 ◊	–
8	-	-	-

9	-	-	-
10	-	-	-

- indicate clear solution no growth, ± indicate standard error of means, n=3, data in the same column marked are statistically significant (p<0.05), cfu/ml indicated as ▲= 10^9, ● = 10^8, ■ = 10^7, □ = 10^6, ○ = 10^5, ◊ = 10^4 etc. Lp, Lr, and La are the (*L. plantarum, L. rhamnosus, and L. acidophilus*) LAB isolated from sheep milk but are grown in the milk of buffalo.

4.4.2 Quantification of organic acid and determination of pH value

The Lp, Lr, and La from sheep milk coagulated the pasteurized buffalo milk and produced organic acids in it, which are detected by titrimetric methods. The results are presented in Table 4.3. The result shows that there is an increase in the percentage of organic acids with a gradual increase in time from 24 to 72 h. the results obtained in the case of all three LAB are observed nearly the same. The decrease in pH is observed with the increase in the percentage of organic acid in case of all LAB. Strain La shows comparatively lower pH 4.1± 0.11 value as compared to all other strains. Thus, there is no significant difference in organic acid production observed in the case of Lp, Lr, and La strains. Increase in organic acid concentration from 2.98 ± 0.21 to 9.17 ± 0.65 helps to reduce the pH value approximately from 6.2 to 4.0 in the surrounding environment of Lp results in the killing of pathogens. The reduction in pH and increase in organic acid production by LAB was found responsible in preventing the growth and manifestation by the pathogens [14].

Table 4.3. Determination of percentage of organic acid and pH value of isolated LAB

Isolates	Incubation time (h)	Incubation temperature (^{o}C)	Organic acid (%)	Initial pH of milk	pH at the end of the incubation
Lp	24	37	2.98 ± 0.21	6.7	6.2 ± 0.11
	48		5.22 ± 0.44		5.4 ± 0.21
	72		9.17 ± 0.65		4.0 ± 0.54
Lr	24		2.51 ± 0.74		6.6 ± 0.41
	48		5.41 ± 0.84		5.4 ± 0.42
	72		9.04 ± 0.15		4.2 ± 0.35
La	24		2.98 ± 0.24		6.2 ± 0.27
	48		4.32± 0.11		5.1 ± 0.24
	72		5.41 ± 0.78		4.1± 0.11

± indicate standard error of means, n=3, data in the same column marked are statistically significant (p<0.05). Lp, Lr, and La are the (*L. plantarum, L. rhamnosus, and L. acidophilus*) LAB isolated from sheep milk but are grown in the milk of buffalo.

4.4.3 *In-vitro* LAB activities against physiological stress conditions

A. Bile test by Lactobacillus

Survival of selected Lp, Lr, and La strains during 3 h period, in the presence of 0.0%, 1.0% and 1.5% bile solution demonstrated that LAB strain Lp shows good survival rate log 110 ± 3 cfu/g at 3 h, while LAB strain La shows the lowest survival that is log 71 ± 1 after 3 h cfu/g. All selected LAB strains show moderate activity at 0% bile concentration up to 3 h of incubation. Comparable to all, the Lp strain shows highest bile tolerance growth followed by other LAB at 1% and 1.5% bile concentration up to 3 h of incubation (Table 4.4). The simulated extreme stomach pH and intestinal condition, i.e. bile test *in-vitro* studies showed that these LAB were with a good survival rate. Currently, the LAB available in the market is sensitive to gastrointestinal stress conditions [15].

Table 4.4. Effect of concentration of bile solution on isolated LAB grown in buffalo milk

Bacterial isolates	Bile concentration (%)	Viable count (log) cfu/g for different % bile solution			
		0h	1h	2h	3h
Lp	0.0	211 ± 3	195 ± 2	189 ± 1	185 ± 3
Lr		199 ± 5	199 ± 2	195 ± 3	191 ± 1
La		190 ± 3	185 ± 2	181 ± 3	180 ± 4
Lp	1.0	210 ± 3	185 ± 2	175 ± 1	170 ± 3
Lr		197 ± 5	189 ± 2	177 ± 3	168 ± 1
La		191 ± 3	181 ± 2	171 ± 3	165 ± 2
Lp	1.5	210 ± 3	165 ± 2	158 ± 1	110 ± 3
Lr		198 ± 5	159 ± 2	151 ± 3	105 ± 1
La		181 ± 4	143 ± 2	101 ± 2	71 ± 1

± indicate standard error of means, n=3, indicate means viable count (cfu/g). Lp, Lr, and La are the (*L. plantarum*, *L. rhamnosus, and L. acidophilus*) LAB isolated from sheep milk but are grown in the milk of buffalo.

B. Cholesterol assimilation test by Lactobacillus

Very few strains of the probiotic are observed with the ability of cholesterol absorption. The *in-vitro* assay help to understand to visualize the cholesterol absorption from the culture medium. This simulation study shows that these LAB cultures may prevent cholesterol deposition in the plasma by its activity in the lumen of the gut. In the present study, 100 µg/ml of cholesterol and (0.2% or 0.4% w/v) concentrations of oxgall were treated with LAB solution. The following study shows that the increase in the concentration of bile also increases the cholesterol assimilation rate. In La, *a* minor increase in the percentage of cholesterol assimilation is observed by an increase in bile salt concentration. In the case of *Lp* and *Lr,* the increase in the cholesterol assimilation is observed significantly by the increase in bile salt concentration.

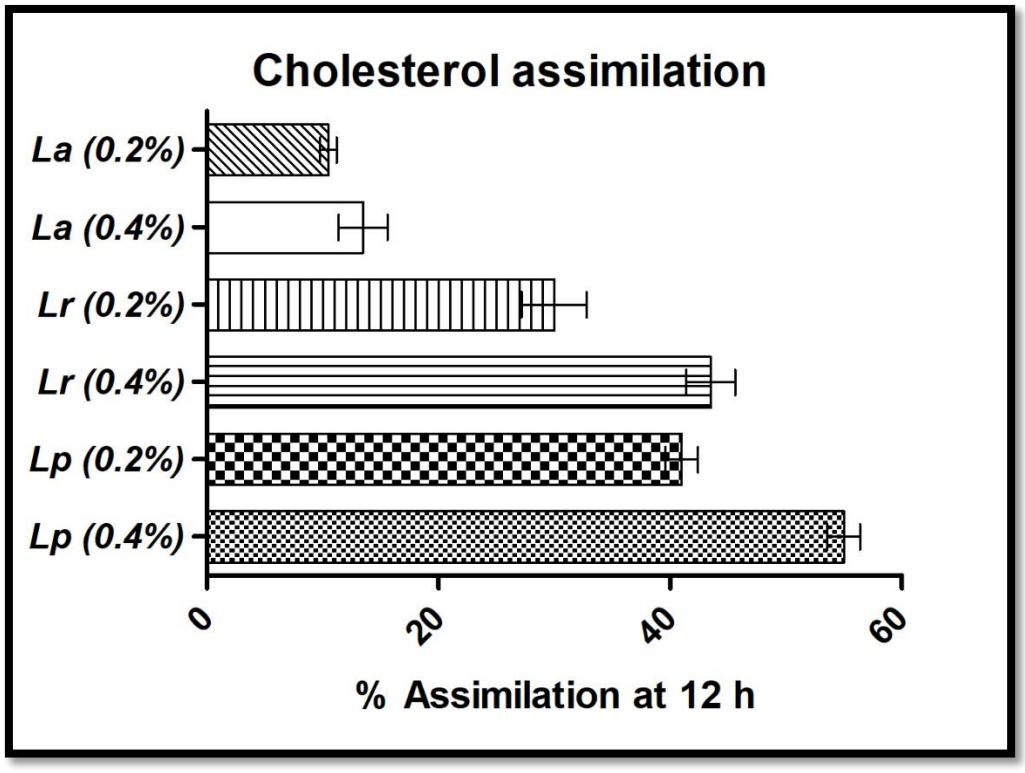

Fig. 4.2. Cholesterol assimilation by the Lactobacillus strains, whereas 0.4% and 0.2% are the concentration of oxgall, ± indicate standard error of means; n=3

The cholesterol assimilation phenomenon is observed only when the cultures were incubated in the presence of bile salts under microaerophilic conditions. The rate of cholesterol assimilation was found directly proportional to the concentration of oxgall (from 0.2% to 0.4%). This study reveals that the increase in bile concentration from 0.2% to 0.4% led to an increase in cholesterol assimilation approximately 29% to 44% for Lr and 42% to 58 % for Lp (Fig 4.2).

C. Lactobacillus resistance against phenol

The Lp, Lr, and La show a greater survival rate not only for the simulated gastric juices but also against phenol. Mostly all three LAB strains showed mixed sensitivity against phenol. Isolate Lp shows good survival and less sensitivity toward phenol, whereas Lr shows medium level sensitivity and La shows higher level sensitivity as compared with the Lp (Table 4.5).

Table 4.5. Resistance to phenol exhibited by the Lactobacillus strains

Isolates	Viable count (log cfu/ml) *	
	0 h	24 h
Lp	7.61 ± 0.03	7.51 ± 0.06
Lr	7.51 ± 0.02	6.11 ± 0.03
La	7.21 ± 0.04	5.01 ± 0.08

± indicate standard error of means; n=3

D. Lysozyme survival by Lactobacillus

Mostly, all three LAB strains showed mixed sensitivity against lysozyme activity. Tested isolates are lysozyme resistant, where Lp shows maximum survival

of approximately 97.61% log cfu/ml, while La shows the least survival of approximately 57% log cfu/ml (Table 4.6)

Table 4.6. Resistance exhibited by the Lactobacillus against lysozyme

Isolates	Resistance against lysozyme log cfu/ml
Lp	97.61 ± 0.13
Lr	92.21 ± 0.21
La	57.22 ± 0.32

± indicate standard error of means; n=3

E. BSH activity by Lactobacillus

The bile salt hydrolysing activity by the LAB are evaluated at high-bile salt concentrations. LAB shows the zone of precipitation indicating the BSH positive strains activity in case of all strains. Lp shows a maximum white zone of precipitation against TGC (Table 4.7). While La shows sensitivity against TDC and TC. Both Lr and Lp show its ability to hydrolyse bile salts. It revealed that these LAB especially Lp is with the ability to deconjugate these salts along with the survival capability by means of intestinal colonization.

Table 4.7. BSH activity exhibited by Lactobacillus strains

Isolates	De-conjugated bile salt		
	▲TGC	●TC	*TDC
Lp	+++	++	++
Lr	++	++	+
La	+	-	-

+++ High; ++ medium and + low activity

F. Survival to SSDP by Lactobacillus

This test was a simulated assay used to study the effects of low pH, bile and different juices of duodenum on different LAB. All the strains show the survival against the above said simulated stress condition. This suggests that LAB may sustain and show the survival in the real micro-environment of the intestine. Lp shows maximum survival rate as compared to other LAB (Fig 4.3). The results show that isolate Lp is with a slight decrease in log count after 3 h exposure to SSDP and with survival of 64.23 % from 79.59% at 0 h, while Lr shows a greater decrease in log count after 3 h exposure to SSDP and also shows moderate survival of 51.21 % from 81.79% at 0 h as compared to La which is showing poor survival.

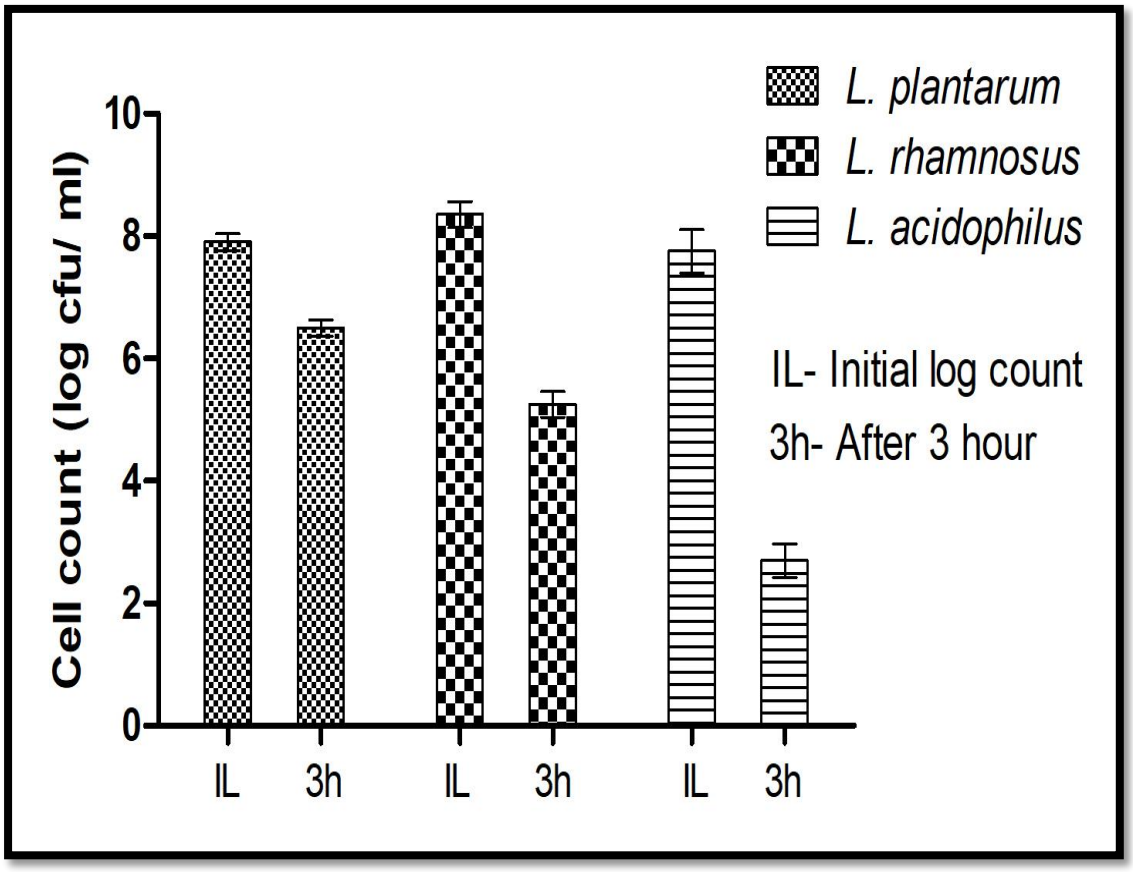

Fig 4.3. Survival of Lactobacillus to SSDP condition, ± indicate standard error of means; n=3

G. Hemolytic and DNAse activities by Lactobacillus

The important properties of an ideal probiotics species are its safety and non-pathogenicity. All the LAB did not show any kind of DNAse and hemolytic activities. It shows that all the isolates are non-pathogenic and are considered safe for host consumption.

4.5 Conclusions

L. plantarum shows a higher survival rate in the gastrointestinal simulation studies as compared to the other two strains of reference. This culture of *L. plantarum* is found more effective in with-holding heavy stress condition like bile salt, pH, lysozyme activity, NaCl concentration with effective sugar fermentation abilities in the host's gastrointestinal tract. Additionally, *L. plantarum* shows the

cholesterol assimilation and negative haemolytic and DNase activity as compared to the other two strains which suggests it as ideal candidature to be functional food by framing it in a desired formulations form.

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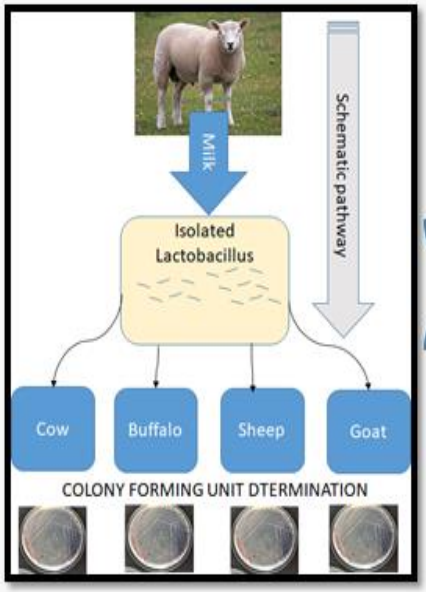
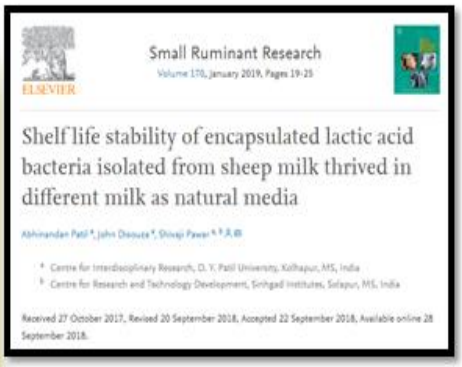
Chapter 5

The part of this chapter have been published as a research article

Studies on flourishing of Lactobacillus in different types of milk and their Lp, Lr and La formulations



Encapsulation techniques



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Shelf life stability of encapsulated lactic acid bacteria isolated from sheep milk thrived in different milk as natural media

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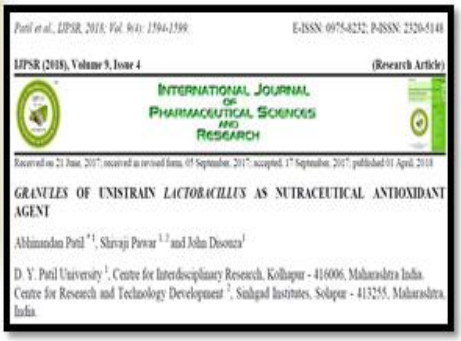

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GRANULES OF UNISTRAIN *LACTOBACILLUS* AS NUTRACEUTICAL ANTIOXIDANT AGENT

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5.1 Introduction

Milk is considered an essential functional food in the human diet. The major source of milk in India, especially in Maharashtra, is milking from buffalo. Along with the quantity, quality of milk, with its contents and compositions makes a lot of impact in dairy industries. One of the most important components of milk as a functional food includes LAB. These bacteria are friendly in nature boosting the natural immune system of the host and is called as probiotics [1]. Milk is found to be the natural media to flourish this LAB. Till date, it was believed that the growth of LAB depends on temperature, pH, and nature of media. Many artificial media are used by the microbiologist for the isolation and culturing of the LAB. It shows that the composition of synthetic media may alter the growth rate of the LAB. Considering this fact, the contents of milk and nutrition is needed to study in case of milking animals. The demand is increased to culture the probiotics potential LAB in the milk of cow, goat, and buffalo; which further significantly helped in the prevention of enteric infection [2]. It is also reported that natural media with a different concentration of the minerals promotes the growth of LAB [3].

In Maharashtra, especially in the region of Kolhapur, Sangli and Satara district, milk of buffalo is favored for consumption as compared to the milk of other milking animals. The content of milk i.e. fat, protein, lactose, and total solid content vary in its concentration in different breeds of buffalo milk. LAB are nowadays flourished in different milking animal's fermented milk and even in fruit-based nutraceutical beverages [4]. Many researchers reported that the protein and total solid content present in the milk of water buffalo are 4.35% and 17.40% respectively while the solid not-fat content value is about 8.7% [5]. Also, the quantification of lactose and casein were determined by scientists [6]. According to 19th 2012 livestock census, 5594 buffalos were reported in Maharashtra by the National Bank of Agriculture and Rural Development Committee, India [7]. In this, Bhadawari, Mehsana and Nagpuri types were found suitable for milk production in Maharashtra state. Bhadawari water buffalo are raised in Agra and Etawah region of Uttar

Pradesh, India with the average milk yield of 800 kgs per lactation. The origin of Mehsana buffalo is from Gujarat, India. The average milk yield is 1300 kgs per lactation. The breed Nagpuri also called as Barari, is from Nagpur, India. The average milk yield of this breed is approximately 1100 kgs per lactation. However, the dairy industries in India not only consider the quantity of milk but also overlook the quality aspects in term of fat, protein, lactose and solid content and even microbial content [8]. Mostly dairy industries use different types of LAB genera as a starter culture for milk products preparations [9]. The growth parameters of these cultures vary as per the type of milk or raw material used for the flourishing. Similarly, in this context, an attempt is made to determine the growth profile of LAB in the milk of different breeds of buffalo's. Thus, the effects of the physiological composition of Bhadawari, Mehsana and Nagpuri milk, i.e. fat, protein and total solid content as a natural flourishing media is investigated to understand the growth pattern of the LAB.

LAB are having a symbiotic association with the existing gastrointestinal microflora preventing the dysbiosis and disease condition. Lp and Lr are considered as the most important lactic acid bacteria conferring health benefits [10]. Due to these functional abilities, nowadays these probiotics are available in different formulation forms [4]. But the most concerning part is its short shelf life; as the formulations deteriorate by a changes in temperature during storage conditions [11]. Many studies revealed that cfu per gram value above 10^8 counts are found to show the health benefits [12]. But it is found that during the storage condition the cfu level falls down below the threshold, resulting in zero pharmacological activities [13]. Thus, many drying techniques are employed to maintain the cfu/g of LAB, making suitable for long-term storage [14]. The spray drying method is found a most favorable technique to generate LAB granules. Spray drying yogurt to preserve *Lactobacillus* and dairy starter cultures have been long investigated [15]. Lyophilized and spray dry probiotic cultures are found to be stable with long-term storage life as compared to tray dry or other drying methods [16]. Yet there are difficulties like low survival rates of the

probiotics during spray drying and poor rehydration properties of the resulting powders [1]. The product integrity of encapsulated LAB depends on the type of microencapsulation techniques and wall materials to be used [17]. Freeze dry or lyophilization is another method to convert liquid LAB to dry powder form. Technological problems such as sudden rise or fall in temperature results in cell mortality or decrease in viability of LAB [18]. These problems are sorted by an introduction of many lyoprotective excipients during drying processes [19]. Maltodextrin, lactose is the most preferred thermo-protective agent used in case of spray dry method [2]. These excipients, not only converts LAB into dry form but also maintains the viability for a long period.

The present study deals with the flourishing of the LAB in the milk of different milking animals. Further, the growth of LAB is monitored in the milk of different local buffalo breeds of Maharashtra. Furthermore, to solve the issue of contamination, LAB is needed to be converted into dry product forms by encapsulation techniques. The encapsulation of the two different LAB isolated from sheep milk is carried out by lyophilization and spray dry coating process. The study is extended to evaluate a shelf life of LAB at two extreme different temperature of 4 ^{o}C and 37 ^{o}C. Further, the investigation is carried out to determine the relationship between the use of different lyoprotective excipients during encapsulation and the factors affecting the LAB viability.

5.2 Experimental

Fig 5.1 represents the general methodology used for the growth of the LAB in the milk of different milking animals.

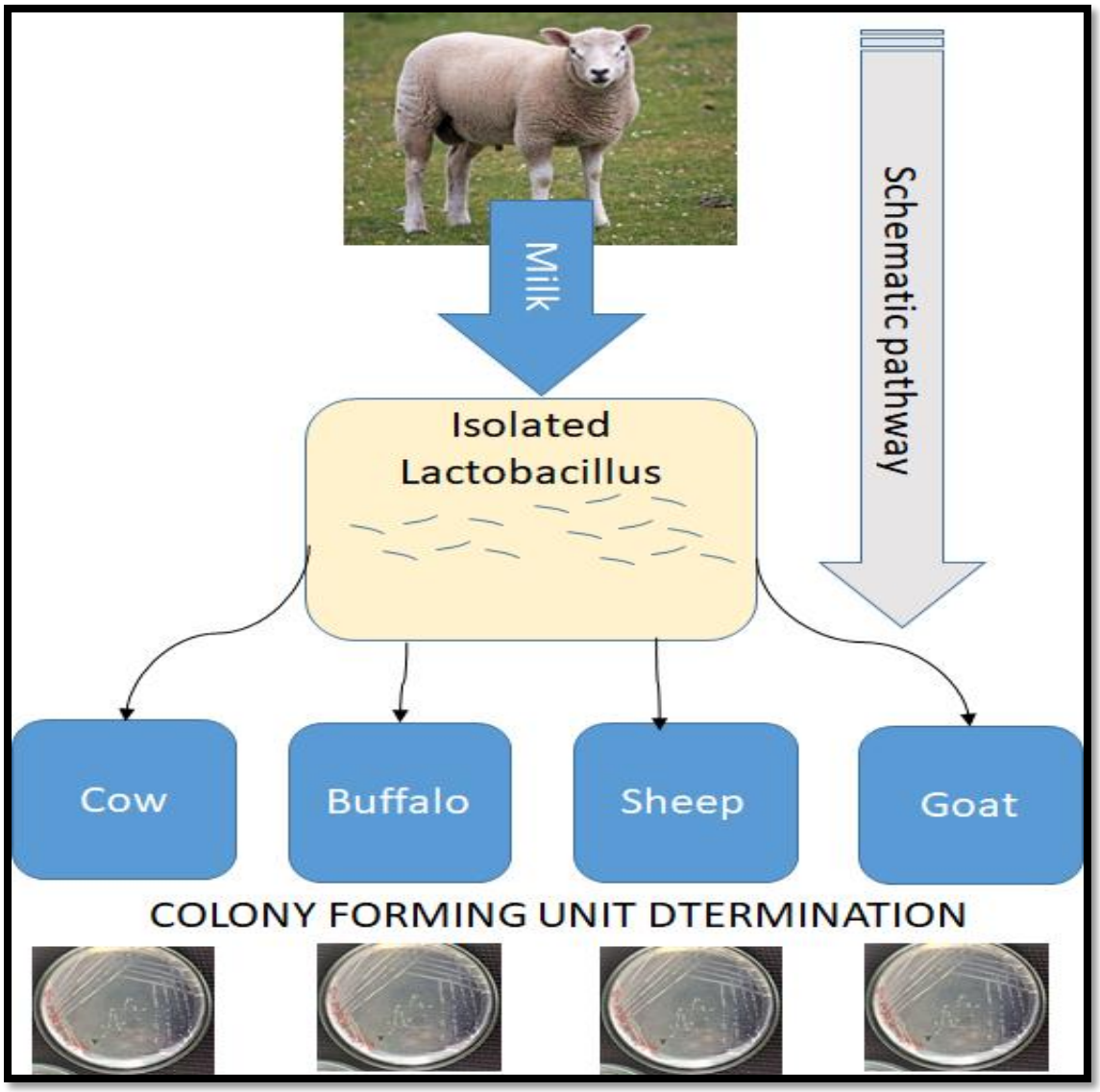


Fig. 5.1. Schematic pathway of isolation and culturing of Lactobacillus in the milk of different milking animals.

5.2.1 Cell preparation and flourishing of isolated LAB in the milk of different animals

The two different LAB isolated from sheep milk using selective MRS, media were incubation for 37 ^{o}C, at 24 h in an anaerobic jar. After incubation, the selected colonies were transferred into MRS broth media at 37 ^{o}C with an adjusted pH of 6.5 for 18 h. Further, these isolated cultures are inoculated in the milk of different milking animals as the natural media for their growth.

The milk of different animals was pasteurized at 75 °C for 15 s and then to ultra-high-temperature (UHT) pasteurization at 140 °C for 3 s before use, in order to nil the other microbes present in the milk samples [20]. The microbes from early MRS media plates were transferred via nichrome lope in aseptic condition to the media of milk of these different animals. Latter these flasks of milk inoculated by microbes of LAB were kept in an incubator at 37 °C for 24 h [21]. The isolated strains of LAB i.e. Lp (A) and Lr (B) from sheep milk; inoculated in the milk of cow were labeled as (A1 and B1). Similarly, the isolated strains of LAB inoculated in the milk of buffalo were labeled as (A2 and B2). The isolated strains of LAB inoculated in the milk of goat were labeled as (A3 and B3). The isolated strains of LAB inoculated in the milk of sheep were labeled as (A4 and B4). The cow, buffalo, sheep and goat milk were obtained from Khillari, Mehsana, Decanni (Mixed colored) and Konkani kanyal breeds respectively. Similarly, the growth pattern of reference standard La (C) obtained from the NCCS (National centre for cell science) was grown in the milk of the cow as C1, buffalo as C2, goat as C3 and sheep as C4 respectively, total 12 sample specimen was characterized by cfu.

5.2.2 Cell preparation and flourishing of LAB in the milk of different buffalo breeds

The milk of buffalo is consumed mostly in all local areas of Maharashtra. Thus, milk of different breeds of the buffalo was evaluated for the more effective growth of the Lactobacillus. A total of 151 milk samples were collected from three breeds of buffalo i.e. Bhadawari (n=50), Mehsana (n=50) and Nagpuri (n=51). The buffalo milk of approximately 40 ml was used for the experimentations. This was further treated by ultra-high-temperature (UHT) pasteurized for 8 s in an alternative cycle for a period of 60 min. These collected milk samples were kept in a sterile 50 ml centrifuge tube at 4 °C before use. The total solid content (%), protein (%), casein (%), density (%), freezing point (%), fat (%) and lactose (%) were determined by FOSS milk scan™ 120 (calibrated with buffalo standards, Warna dairy, India) milk analyzer. The isolated LAB from MRS Broth was transferred via nichrome lope in

the milk of these different buffalo breed and incubated; evaluated for its cfu count and consistency.

5.3 Different coating excipients for Lactobacillus and their encapsulation techniques

A) Granulation/ agglomeration by different drying methods

Various encapsulation techniques are employed to generate the granule formulation. The morphological forms developed during drying processes are of different shapes such as irregular, simple, matrix, multi-wall and multi-core forms (Fig 5.2).

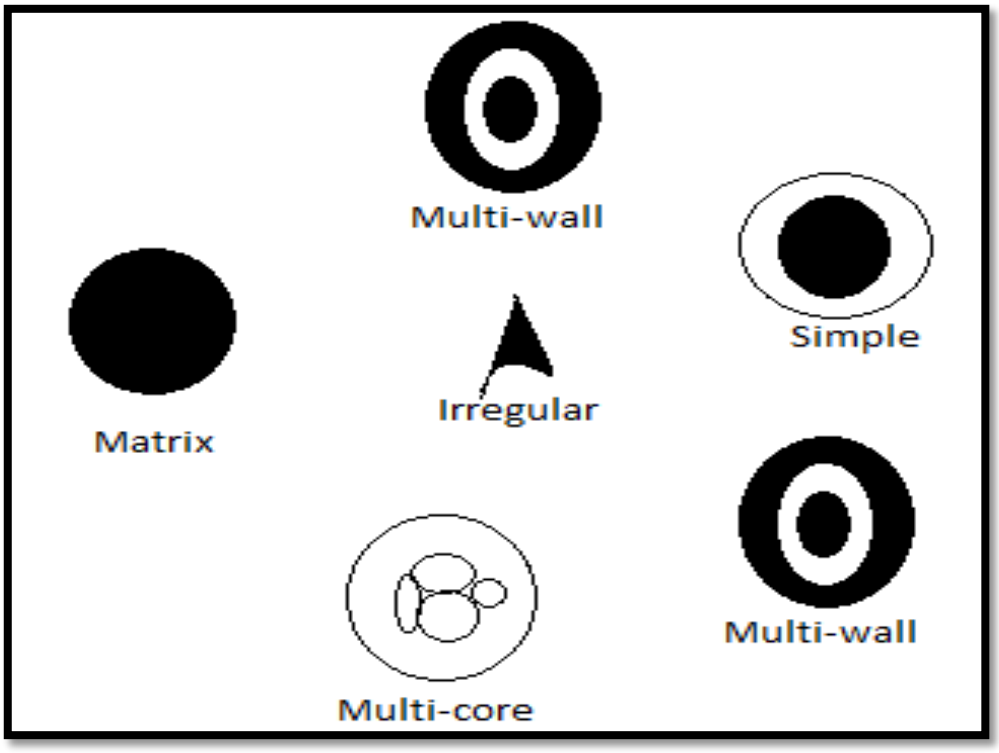


Fig. 5.2 The different form of agglomerates developed during the encapsulation process

The probiotics encapsulation techniques currently available in the pharmaceutical industries include methods such as spray-coating, freeze dry, spray coating and spray drying technology. The spray coating method involves the spraying of the coating materials on the active pharmaceutical ingredients in unidirectional motion. In the spray dry process, the active pharmaceutical ingredient along with the thermoprotective materials are sprayed by a small bore into a heated

chamber generating the small fine agglomerates [22]. In industries, emulsion and extrusion techniques were used for a long time for the bacterial encapsulation with a view to preventing the cellular injury. On contradictory, spray coating and spray drying are used rarely due to their high cost and optimization issues. However, spray drying with the proper batch optimization and excipient ratios found in producing high capacity products at very low costs [23].

B) Spray drying technique

The method of the spray dry produces the dehydrated optimized biological materials at a low cost. Commercially many probiotics formulations are produced at the large by this technology. The mechanism of the spray dry involves the atomization of the concentrated liquid into the small droplets by a nozzle at high pressure into a hot drying chamber. The high temperature removes the water content rapidly producing the dried agglomerates collected in the sterile bag. The product drying in a spray dryer is a two-stage mechanism. The first drying step is described as a constant heating phase which involves the heat at the wet outer droplet surface [24]. The temperature in this phase is around 50 °C, the same as the air's wet-bulb temperature but lower than the air temperature (110-180 °C). This temperature protects the bacterial cell surface and maintains its integrity. The wet bulb temperature around the particle or encapsulated bacteria protects it from the external high-temperature inlet heat. Thus, the particle while passing through the heating chamber loses its water content without the exposure of heat to the particle or bacterial cell wall. The actual heat experienced by the particle is the same as that of the actual outlet temperature and not of the inlet heat. Apart from the heat, the excipient used to coat the particle called thermo-protective materials play a vital role in maintaining the cellular integrity of the bacteria. The rate of bacterial survival depends not only on the scale-up and optimization of the batch but also upon the use of excipients with its combination [25]. The real challenge is the identification of the excipient and its combination for the cell protection with the longevity of the final product by the optimisation process.

C) Spray drying of probiotic bacteria

The spray drying is the most recommended methodology in the industries for the drying of the probiotics due to its high productivity and low-cost operational inputs. The spray dry method provides the good quality dried probiotic formulation with the consistency in particle size and free-flowing abilities [25,26].

The culture media along with the thermo-protective materials and cell concentration in cfu, determines the longevity of the final finished products. Many researchers, carried out the spray drying of the probiotics at the outlet temperature of 70-90 °C as optimal [14]. However, they reported that the viability of encapsulated microbes decreases by increasing the outlet heat. The studies revealed that excessive outlet temperature fractures the particles exposing the cell surface to the surrounding environment damaging the integrity of the cells. The heat-sensitive pharmaceutical products require very low outlet heat conditions [26,27]. The long term shelf life of the finished dried product is achieved if the moisture content of products is kept below 5%. The researcher found that the feed solution and its solid content in it along with the feed flow rate affect the particle size of the end encapsulated products.

D) Lyophilisation

Lyophilisation is the most preferred method for the biological materials in the pharmaceutical and food industries. It is the most sensitive and reliable method to generate the biological material into dry solid form. The classical freeze-drying process is a technique in which under a reduced pressure the solution is frozen by various subsequent freezing technique to the dried particles. In this drying technique, the integrity of the biological product is maintained in physical and chemical structure with the intactness of the biological activity. The bio-pharmaceutical products assure the safety and the product sterility during the lyophilization of products in vials or syringes [28]. The contamination free pharmaceutical product is easily derived by this method as compared to other drying techniques. The change of production of biological products from batch to continuous or vice-versa is easily

maintained by the freeze-drying method (Fig 5.3). It mainly consists of three basic steps i.e. product freezing, primary and secondary drying [29]. The water is crystallized and is separated from the sample by the freezing process. In the next step i.e. primary drying, the frozen solid is sublimated to a gaseous state. Finally, in the secondary drying step, the non-frozen part is removed by desorption and the dried products is obtained.

E) Lyophilisation of probiotic bacteria

Freeze-drying or lyophilization is the most generalized method employed for the production of probiotics agglomerates in dried forms. The uses of the cryoprotectants or lyo-protectives during the drying processes increases the viability of cells after drying. Moreover, a variety of cryoprotectants have been used for lyophilization of probiotics in order to increase the survival rate of microorganisms [29]. Many natural and synthetic origin lyoprotectants excipients such as skim milk, sucrose, lactose, trehalose, and whey protein were studied to coat these friendly microbes. The combinations of these excipients in different ratios and proportion were found as a more effective drying agent as compared to their individual uses. Earlier experimentation demonstrated by the researchers showed that the use of skim milk along with the sodium ascorbate enhanced the shelf life of the *L. paracasei* for more than 3 months when stored at 4 ^{o}C [28]. Similarly, scientists found various effects of the skim milk and the trehalose on the shelf life stability of the *L. rhamnosus* for a period of 3 months at 4 ^{o}C and observed the survival rate above 94% [30]. The researcher investigated the role of a pharmaceutical excipients such as fructose, ascorbic acid and skim milk on encapsulation of the *L. gasseri* and found survival rate of around 90% after 120 days [31]. They further investigated that the absence of the protective agent reduces the shelf life below 70%.

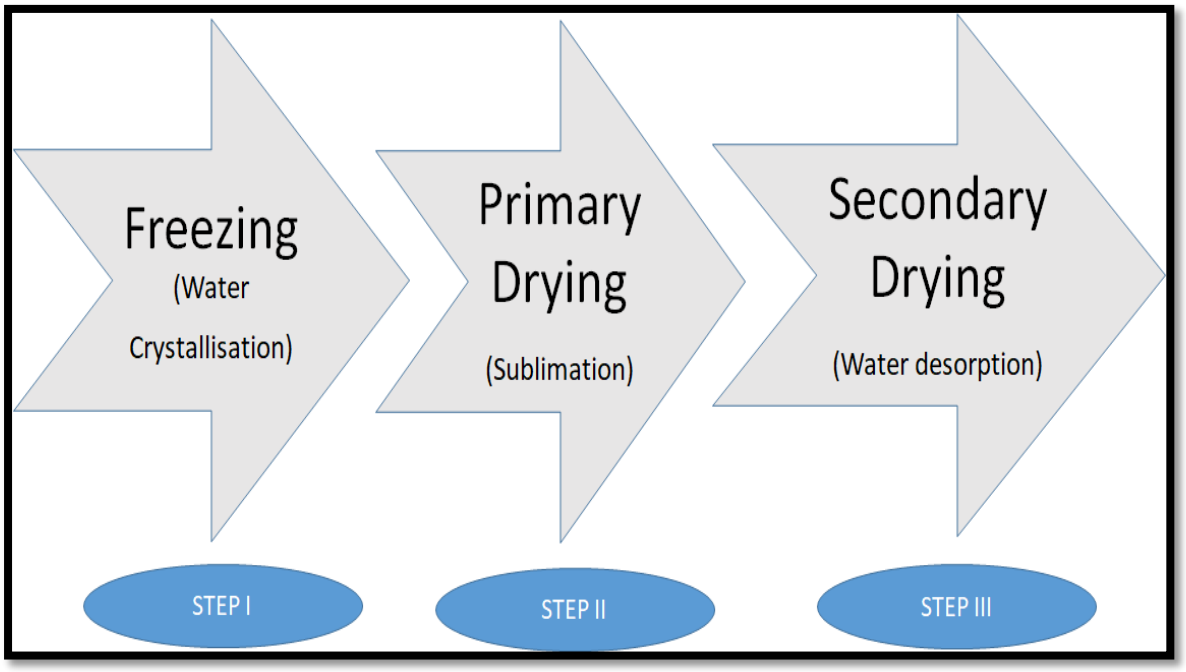


Fig. 5.3 Different steps involved in the lyophilization process

F) Effects of encapsulating materials on drying technique

The coating materials used for the encapsulation of the probiotics prevent the degradation of the products and maintains its quality during distribution and storages. Apart from the encapsulation, this coating material also controls the release of the probiotics at target area i.e. the villi of the intestinal tract. Many natural and synthetic origin coating materials were used to protect the probiotics from the harsh micro-environment of the intestinal tract which includes versatile pH and different physiological conditions [17]. The most favorable coating materials used are carbohydrates. These carbohydrates include the use of maltodextrin, starch, and sugars. Proteins and hydrocolloids are also found as an effective thermo-protective agents to encapsulate the biological products and microbes [32]. These materials maintain the cellular integrity and enhance the survival rate of the finished coated materials. The most important part of coating material is that it should be free from the side effects such as delayed cell recovery and viability.

a) Carbohydrates and sugars

Researchers and industrialists are using carbohydrates as an ideal source of a coating or encapsulating materials for spray drying and lyophilization processes. Increase in concentration of the feed solution above 20% as coating solution not affect the viscosity and final product integrity. Maltodextrin is used as bulking and coating material in case of both spray drying and lyophilization processes [33]. These sugar coatings are found effective to increase the stability of probiotics by enhancing the integrity of cellular material by binding to the phospholipid bilayers membrane of the cells in order to increase the cellular viability. The sugars such as glucose, sucrose, and trehalose were found as thermo-protective and lyo-protective agents for coating LAB [34]. The current showed that the use of these types of sugar as growth media or as drying agents increases the cell viability with long term shelf life.

The water around the cells is replaced by these sugars during the coating process, forming a glassy transformation around the cell surfaces. The cellular protein of the probiotics during the drying process binds with the hydrogen bonds of the sugar, thus enhancing the cellular integrity. Further investigation proved that the use of these sugars does not interact with the polar head of the protein thus preventing the disruption of the cellular materials. Similarly, the trehalose is used as an effective coating agent as compared to the other sugars due to its ability to penetrate the bilayer lipoprotein during the drying processes [32–34]. The use of the polysaccharides such as starch and its derivatives along with the dextran acts as a protective agent and are found cost-effective as compared to disaccharides. However, large chain structure of these polysaccharides fails to maintain the long term shelf life when used alone as a feed solution. It is mostly recommended to use the combination of polysaccharides along with the monosaccharide's to maintain the integrity of the end products. These sugars act as a cryoprotectant agent in the lyophilization process. No any kind of osmotic stress is observed during lyophilization process by use of these types of sugars.

b) Proteins

Alike carbohydrates, proteins are also recommended as a coating material for probiotics by a spray dry and lyophilization process. Skim milk and milk proteins are found effective coating agents due to it's both hydrophilic and lipophilic nature by showing higher binding properties to the cell membrane. Proteins are found as the most effective glass coating agent around the microbes with higher glass transition membrane temperature. Mostly, skimmed milk is used in the combination of sugars to get maximum viability and shelf life [17]. Single use of protein for coating is found ineffective due to denaturation problem during the heating process. Many scientists demonstrated that an increase in the concentration of the skim milk as a feed and coating material, increases the survival rate of encapsulated probiotics during the spray dry process. Many researchers carried out the spray drying technique for *Bifidobacterium* coating using gelatin, starch and skim milk [35]. They evaluated that the results observed in case of the combination of the skim milk and starch as a coating material for probiotics showed higher viability as compared to individual use of these thermo-protective agents.

5.3.1 Lactobacillus cell preparations and different drying techniques

5.3.1.1 Spray drying of LAB

The two different probiotics i.e. Lr and Lp were inoculated in MRS broth (MRS, Siffin Pharma, Germany) for 20 h at 37 ^{o}C. To generate the microbial load, these cells were grown in micro-aerophilic condition using UHT skim milk. All the cells were grown till early stationary phase at 37 ^{o}C for a period of 14 h till semi-solid mass is formed. The semi-solid mass generated were homogenized at 5500 rpm for 10 min at 4 ^{o}C using Tetra pak aseptic homogenizer (Fig. 5.4).

Fig. 5.4. Tetra pak aseptic homogenizer

Fig. 5.5. Spray dry equipment (JISL Mini-spray)

A. Spray dry of LAB thrived in buffalo milk and shelf life determination

The excipients used for the spray dry techniques were lactose, starch and maltodextrin. The combinations of lactose and starch (2:1) were used as the thermo-protective agent to prevent the mortality of the microbes. The encapsulation of Lp, Lr, and La at different combinations of maltodextrin and starch solution in ratios 1:1, 1:2, 2:1 were carried out to gain good viability [17]. This resulted in the formation of semi-solid masses. The generated semi-solid masses of LAB strains along with the different combination of above said excipient were kept at 4 ^{o}C prior to the encapsulation process. To process it further, these semi-solid beads along with excipient combinations were again homogenised from semi-solid mass into a liquid state by REMI homogenization (RQT-127 A/D Digital - Homogenizer, Motor-AC/DC 1/8 HP). Rotation of around 2300 rpm was used to break the semi-solid formed masses [1,17].

The spray-drying process of LAB using the selected natural media was undertaken in a laboratory scale JISL mini-spray dry (Laboratory spray dryer LSD-48, India) (Fig. 5.5). The feed solution was pneumatically atomized into a vertical, concurrent drying chamber using a two-fluid nozzle at a constant flow rate of 5 ml/min to 20 ml/min. The outlet temperature was adjusted from 100 to 110 ^{o}C by varying the air inlet temperature. Finally, the product is obtained as the dried powder in the collecting chamber. The encapsulated LAB were plated time to time on MRS agar media for a period of 12 months to study the shelf life (viability) at two different temperature of 4 ^{o}C and 37 ^{o}C respectively [36].

5.3.1.2 Lyophilization technique for Lactobacillus

Fig. 5.6. Lyophilizer (Freeze dryer)

The three different probiotics i.e. Lr, La and Lp were inoculated in MRS broth (MRS, Siffin Pharma, Germany) for 20 h at 37 ^{o}C. To generate the microbial load, these cells were grown in micro-aerophilic condition using UHT skim milk. All the cells were grown till early stationary phase at 37 ^{o}C for a period of 14 h till semi-solid mass is formed. The semi-solid masses generated were homogenized at 5500 rpm for 10 min at 4 ^{o}C using REMI homogenizer. The liquid cell suspension were re-suspended in different lyoprotective agents (w/v) i.e. 10% maltodextrin, 10% lactose, 10% sucrose, 10% maltodextrin + 5% sucrose, 10% lactose + 5% sucrose prior to lyophilisation, where skim milk was kept as control.

The cell suspensions along with the excipients combinations were frozen at -20 ^{o}C for a period of overnight in a freeze dryer (U-TECH, star scientific equipment, India) (Fig. 5.6) [28]. Further, the vials were incubated at -70 ^{o}C for 1 h and late freeze dried at -50 ^{o}C at 110 millitorr chamber pressure of condenser for 48 h [26]. The cells

obtained were dried and packed in a plastic container at 4 ^{o}C i.e. with the refrigeration and also at 37 ^{o}C (room temperature) of Indian climatic condition for a period of 12 months [28]

A. Cell viability determination of lyophilised formulations

The cell viability of the LAB formulations were determined by the serial dilution technique on MRS media. The cell count of the prepared samples before lyophilization and after encapsulation were determined by cfu count [37]. Both the type of sample i.e. before and after lyophilization were rehydrated in a 1 ml solution of 5% dextrose aliquot for 20 min at 37 ^{o}C with gentle shaking. The above said samples were plated on MRS media at 37 ^{o}C for a period of 24 h in microaerophilic conditions. The cell viability during the long-term storage for 12 months was calculated and expressed as viability storage factor (Vsf). This calculation of Vsf was calculated as per equation (5.1)

$$Vsf = 1 - (\log cfu_0 - \log cfu_X)/ \log cfu_X \times 10 \quad (5.1)$$

where cfu_0 = initial cfu/g X total weight of dry sample (g),

while cfu_X = (0, 1, 2 . . . n value in hours) time cfu/g X weight of dry sample (g). The calculation was multiplied by 10 to get the value of Vsf in integrals and to avoid the values in decimal.

5.3.2 Evaluation of different encapsulated formulations properties

5.3.2.1 Characterization of the spray dried LAB granules

Optimized formulations (F4, F8 and F12) were firstly evaluated for its flow properties using various characteristics parameters such as bulk density, tapped density, Hauser's ratio, and compressibility index [26,38]

a) Bulk Density

Apparent bulk densities (ρb) of granules were determined by pouring the granules into a graduated cylinder. The bulk volume (V_b) and the weight of granules (M) were determined. The bulk density was calculated using equation 5.2.

$\rho_b = M / V_b$ (5.2)

b) Tapped Density

Apparent tap densities (ρ_t) of granules were determined by the measuring cylinder containing a known mass of blend by tapping 50-125 times using tapped density apparatus (Electro lab, ETD-1020, Mumbai, India). The constant minimum volume (V_t) occupied in the cylinder after tappings and the weights (M) of the blend was measured. The tapped density (ρ_t) was calculated using the formula as in equation 5.3.

$\rho_t = M / V_t$ (5.3)

c) Compressibility Index

The measurement of the flow of the powder can be determined in the ease way by compressibility index (I). The material flow is expressed as compressibility index (I) which was calculated as in equation 5.4

$I = \rho_t - \rho_b \times 100 / \rho_t$ (5.4)

Where, ρ_t = Tapped density; ρ_b = Bulk density

d) Hausner Ratio

Hausner ratio (HR) is an indirect index to determine the powder flow. It was calculated by the formula as shown in equation 5.5.

$HR = \rho_t / \rho_b$ (5.5)

Where ρ_t indicate the tapped density and ρ_b means bulk density.

e) Angle of repose

The angle of repose defines the inter-particulate friction and the resistance occurred in the movement between particles. It was calculated by the formula as in equation 5.6.

Formula, Angle of repose (θ) = $\tan^{-1} h/r$ (5.6)

In this technique, a funnel with a height of 2-4 cm is maintained from the top of the powder pile and is allowed to freely flow. The base of the powder should be free of vibration.

f) Loss on drying

This test was performed as a part of the in-process quality control tests at various stages during the manufacturing process. The instrument used for this purpose was Halogen Moisture Analyzer. About 1.5 gm of sample was placed in the chamber of the instrument and it was kept at 110°C on auto mode. The percentage loss on drying was noted down.

5.3.2.2 Solubility of spray dried and lyophilized formulations

The solubility (%) was determined by equation (5.7) described by Saikia et al. [39]. Briefly, the spray dried powder was mixed with distilled water (1:10 w/v) and stirred for 1 h at room temperature. The mixture was then centrifuged at 3000 x g for 5 min. After that, the supernatant was collected, dried and weighed. The solubility was determined by equation 5.7.

Solubility= Weight (g) of the supernatant after drying/ weight of the sample (g) X 100 (5.7)

5.3.2.3 pH and titratable acidity (TA) of the spray dried and lyophilized powdered samples

The pH of the samples was measured using a pH meter (Eutech, Merck). Briefly, 1 g of sample was dissolved in 5 ml deionized water and pH was measured at 27°C. Titratable acidity (TA) was determined by titration method [39]. 1 g of sample was dissolved in deionized water, 2-3 drops of phenolphthalein indicator was added and titrated against 0.1N sodium hydroxide. Titratable acidity was expressed as citric acid equivalent (equation 5.8).

Titratable acidity (TA) = Titrate value X NaOH (0.1N) X 64 X 100/ Volume of sample used X sample weight X 1000 (5.8)

5.3.2.4 Hygroscopicity of spray dried and lyophilized formulations

Hygroscopicity of the spray dried powders were determined following the method described by Saikia and Corke [39,40] with some modifications. Briefly, 2 g of spray dried powder samples were placed in a pre-weighed glass vial and placed in an airtight desiccator containing saturated sodium chloride solution at 30 ^{o}C, kept for 7 days. After the incubation period, sample vials were weighed. The hygroscopicity was expressed as grams of adsorbed moisture per 100 g of the samples.

5.3.2.5 Scanning electron microscopy study of encapsulated lactic acid bacteria

The encapsulated LAB was observed using the scanning electron microscopy, in which the size of the particles was evaluated.

5.4. Results and Discussion

5.4.1. The growth pattern of Lp, Lr, La in different milk samples

The LAB isolated from sheep milk were grown on MRS agar media at 37 ^{o}C for 24 h. These bacteria are identified as the LAB which is labeled as A for Lp, B for Lr, and C for La, respectively. The experiment is performed by considering La as a reference standard for the comparative analysis [41].

Table 5.1. cfu/ml count growth parameters of LAB in natural media (milk of different milking animal) after 24 h

Isolated LAB from Sheep milk	Milk of Milking animal	Nomenclature	cfu/ml count n=3*
A	Cow	A1	10* X 10^9 cfu ± 2.5
	Buffalo	**A2**	**12* X 10^{10}** cfu ± 7.5
	Sheep	A3	14* X 10^9 cfu ± 2.8
	Goat	A4	04* X 10^9 cfu ± 5.5
B	Cow	B1	24* X 10^9 cfu ± 3.7
	Buffalo	**B2**	**14* X 10^{10}** cfu ± 4.6
	Sheep	B3	18* X 10^9 cfu ± 5.4
	Goat	B4	04* X 10^9 cfu ± 6.8
C	Cow	C1	19* X 10^9 cfu ± 7.4
	Buffalo	**C2**	**11* X 10^{10}** cfu ± 5.2
	Sheep	C3	15* X 10^9 cfu ± 4.7
	Goat	C4	09* X 10^9 cfu ± 6.6

A and B are the isolated microbes from sheep milk; *Lactobacillus plantarum, Lactobacillus rhamnosus,* and C as *Lactobacillus acidophilus* (reference standard) respectively, ± indicate standard error of means, n=3, data in the same column are statistically significant (p<0.05)

All the three LAB from MRS media transferred to UHT pasteurized milk of a cow, buffalo, sheep, and goat shows the growth rate in the range of 10^9 - 10^{10}

cfu/ml after 24 h. Finally, the cfu/ml is found higher in the case of buffalo milk (A2- 12* X 10^{10} cfu ± 7.5, B2- 14* X 10^{10} cfu ± 4.6 and C2- 11* X 10^{10} cfu ± 5.2) as compared to other milking animal milk which follow in range of 10^9 cfu presented in table 5.1. The milk of buffalo is having high iron content (2·40 ± 0·2056 mg/l) and folic acid content (14.6 μg/l) which is very much important for the growth of LAB as compared to the milk of different animals [3]. Previous studies have pointed out that prebiotics are important for the growth of LAB [42], but our studies show that UHT pasteurized milk may act as a good natural flourishing agent [4].

5.4.2 Chemical composition of different buffalo's milk and study of LAB growth pattern

The various nutrient contents i.e. protein, fat, lactose, total solid content and percentage of casein in the raw milk of Nagpuri, Mehsana and Bhadawari are 4.5 ± 0.51, 5.4 ± 0.62, 4.8 ± 0.41; 7.2 ± 0.32, 6.1 ± 0.66, 7.0 ± 0.84; 5.4 ± 0.65, 6.2 ± 0.65, 5.1 ± 0.54; 17.2 ± 1.21, 18.8 ± 1.54, 18.1 ± 1.22; 10.8 ± 1.20, 11.4 ± 1.02, 11.5 ± 0.98; respectively. No any significant (p<0.05) changes are observed in the nutrient contents of raw and pasteurized milk of all breeds as observed in fig 5.7, 5.8 and 5.9 respectively. The protein and lactose content of Mehsana milk (p<0.05) are found to be significantly higher as compared to the milk of the other two breeds. On the contrary, the fat content of Mehsana buffalo are found to be significantly lower (p<0.05) as compared to the milk of Nagpuri and Bhadawari respectively. (Fig 5.10.A)

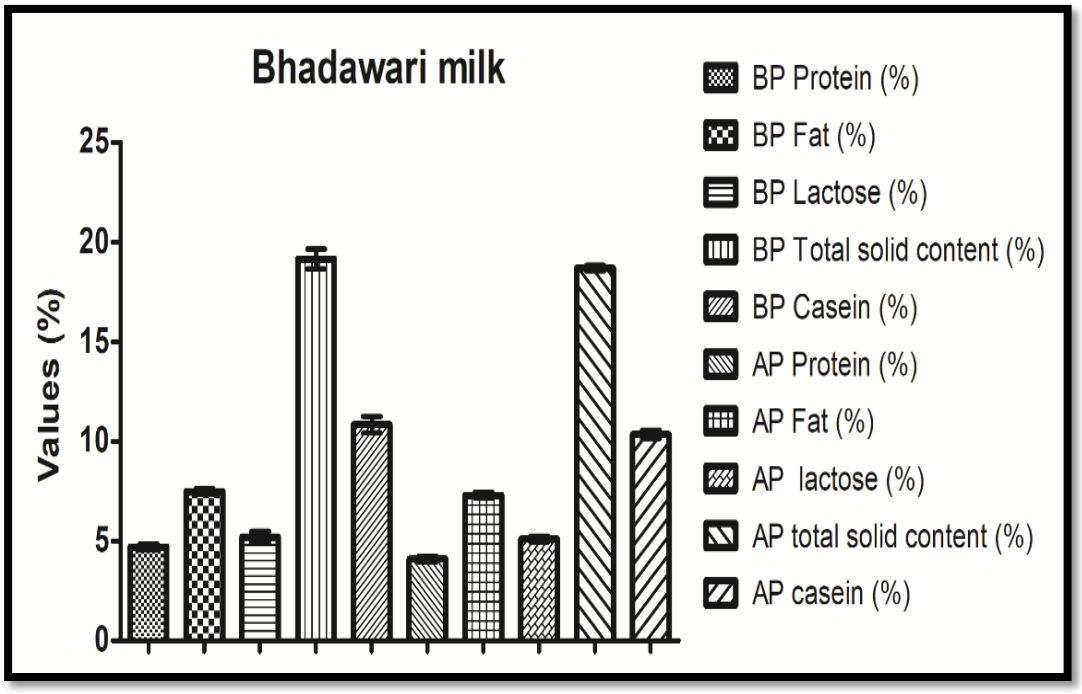


Fig 5.7. The physiochemical composition of Bhadawari milk

± indicate standard error of means; n=3

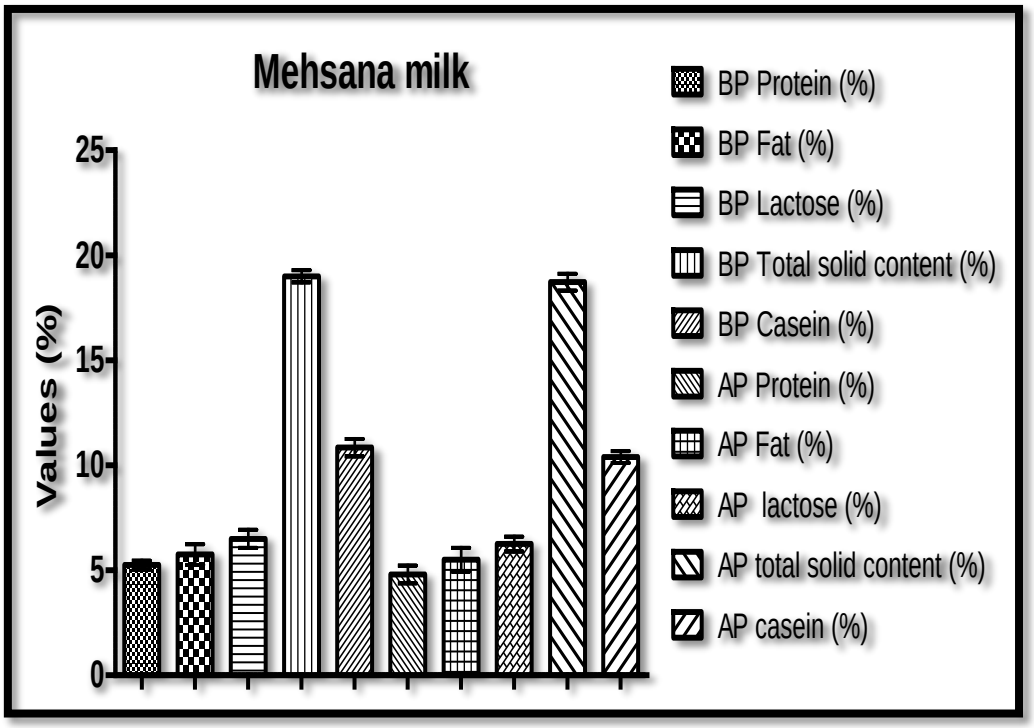


Fig 5.8. The physiochemical composition of Mehsana milk,

± indicate standard error of means; n=3

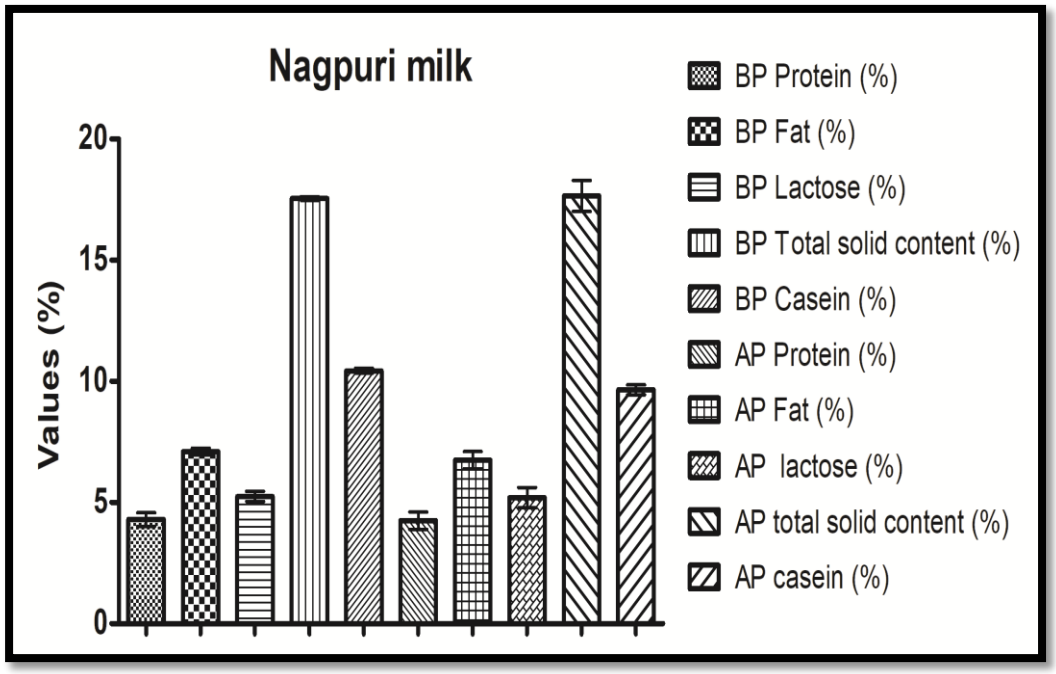


Fig 5.9. The physiochemical composition of Nagpuri milk

± indicate standard error of means; n=3

The current study shows that lactose content in milk of Mehsana is higher 6.2 ± 0.65 than previously reported milk studies of other milking animals [43]. Many other reports demonstrated that the lactose content of Nagpuri and Bhadawari is similar to the Anatolian water buffalo [6]. The fat content observed in the case of Nagpuri and Bhadawari is found to significantly higher as compared to previously reported results [43].

In the case of Mehsana milk, the total solid content of 18.8 ± 1.54, protein 5.4 ± 0.62 and lactose 6.2 ± 0.65 are found higher than the previously reported values [7]. The casein content reported in the case of all Indian breeds is found nearly the same. The cfu count was determined using serial dilution technique till 10^9 times by plate culture method. The analysis of cfu/ml was carried out in the milk of Mehsana, Bhadawari, and Nagpuri. The difference in the cell count is observed with the highest cfu count 10^9 in the milk of Mehsana ($p<0.05$) as compared to the milk of Nagpuri and Bhadawari showing the cell count in range $10^{8\sim9}$ (Fig 5.10.B). Moreover, these LAB flourished in the milk of Mehsana are found to be higher than the milk of other

water buffalo [3]. Many researchers found that LAB can be flourished in fruits juice [36]. But the current study shows that LAB growth is highest in Mehsana milk as compared to other milk or other natural media used for its thriving. The reason for the growth may be the higher lactose, protein and total solid content in milk of Mehsana as compared to the other breeds milk.

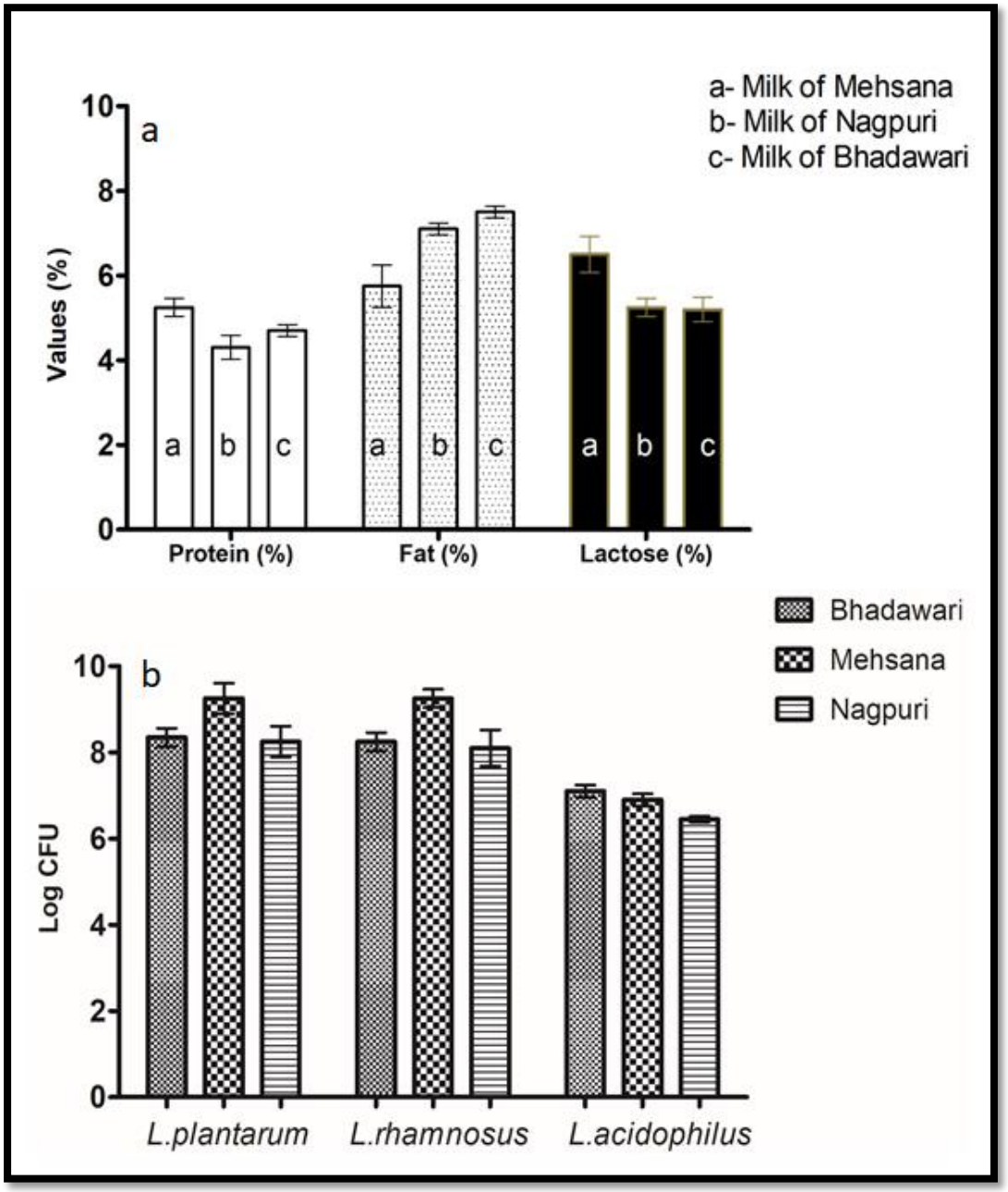


Fig 5.10. A) Protein, fat and lactose contents and B) the log cfu count of the LAB in the milk of different breeds of Indian buffalos

Thus, the current study shows that the quality of milk in terms of lactose, total solid content and protein, affects the growth of LAB. These nutrient values are found higher in the milk of Mehsana milk as compared to Bhadawari and Nagpuri. Thus, Mehsana milk is found suitable for the flourishing of the LAB. Additional, other studies are needed to be done to evaluate the other factors responsible for the growth of LAB in the milk of Mehsana buffalo.

5.4.3 Spray dry of LAB using excipients and their survival studies after encapsulation

The spray dry technique using different excipients enhanced the shelf life of the products. The cfu/ml study relieved negligible change in cell count after encapsulation which was determined by a general serial plate count method (Table 5.2). The following observation showed that maltodextrin and starch in ratio 2:1 with the good cfu/g count. The cfu/g values obtained in case of selected (A2, B2, and C2) LAB by using maltodextrin and starch in ratio 2:1 are $9.7 \pm 0.15 \times 10^{10}$, $9.6 \pm 0.12 \times 10^{10}$, $9.5 \pm 0.14 \times 10^{10}$, $9.5 \pm 0.24 \times 10^{10}$ respectively. The data shows that during encapsulation there is a negligible decrease in cfu/ g count in case of all LAB strains. The different shelf life study profile was observed in the case of encapsulated LAB formulation at 4 ^{o}C and 37 ^{o}C. Combination of maltodextrin and starch in ratio 2:1 shows greater viability as compared to other pharmaceutical excipients in combination for all LAB strains (Fig. 5.11). Lactose and starch combinations show the least viability during 12 months studies at 4 ^{o}C as compared to rest other excipients used, while no growth are observed after 6 months study at 37 ^{o}C (Fig. 5.10) when carried out by serial dilution technique. Maltodextrin and starch in the ratio (1:1) show initially good viability count till the period of 4 months same as other excipients combinations but shows severe decline till 10 months studies at 4 ^{o}C; in case of all LAB strains (Fig.5.11). Maltodextrin and starch in the ratio (1:2) show a dramatic decline in percentage viability after 4 months, but better survival results after 12 months studies than (1:1) combination of the same excipient in case of all LAB at 4 ^{o}C.

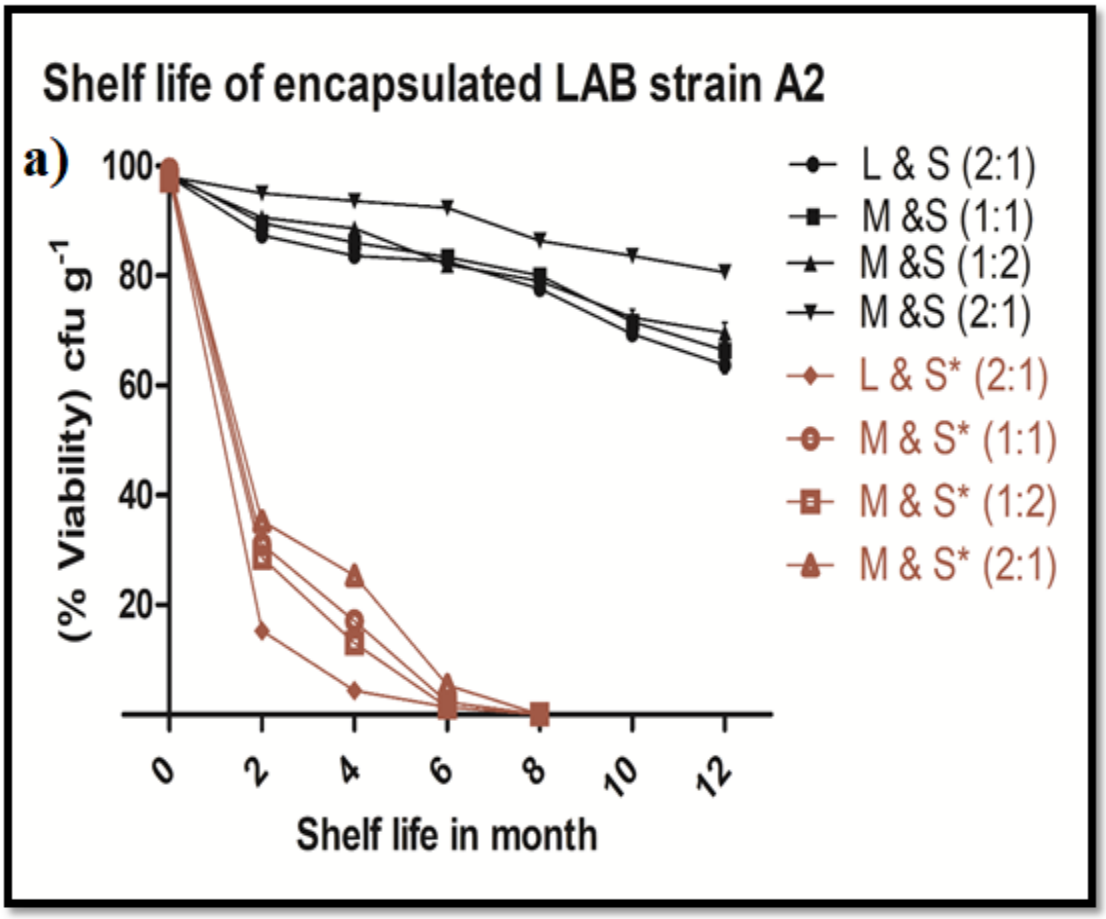
Table 5.2. Spray dry encapsulated excipients of LAB grown in buffalo milk and its cfu/ml count.

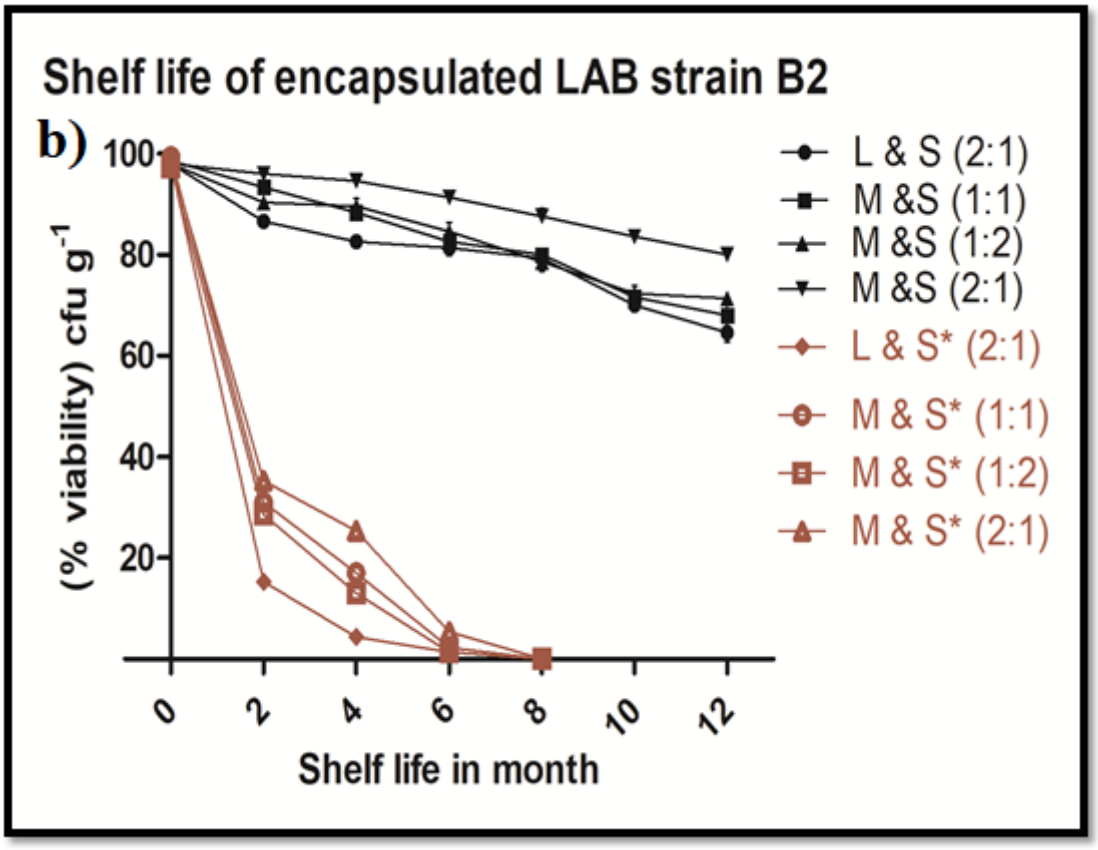
LAB Strains	Nomenclature	Condition	log cfu/ml (*X10^{10})[a]		Yield [b] (%)
			Before E	After E	
A2 (Lp)	F1	Lactose and starch (2:1)	7.1 ± 0.19	6.8 ± 0.19	61.1 ± 1.2
	F2	Maltodextrin and starch (1:1)	9.2 ± 0.24	8.5 ± 0.17	43.5 ± 2.1
	F3	Maltodextrin and starch (1:2)	9.5 ± 0.27	8.9 ± 0.11	55.9 ± 2.9
	F4	**Maltodextrin and starch (2:1)**	**9.9 ± 0.05**	**9.7 ± 0.15**	**61.8 ± 4.1**
B2 (Lr)	F5	Lactose and starch (2:1)	7.2 ± 0.24	6.7 ± 0.14	58.6 ± 5.9
	F6	Maltodextrin and starch (1:1)	7.8 ± 0.09	8.7 ± 0.17	58.8 ± 4.2
	F7	Maltodextrin and starch (1:2)	9.4 ± 0.10	9.1 ± 0.16	71.6 ± 3.2
	F8	**Maltodextrin and starch (2:1)**	**9.9 ± 0.24**	**9.6 ± 0.12**	**69.7 ± 1.5**
C2	F9	Lactose and starch (2:1)	8.7 ± 0.21	7.7 ± 0.14	70.4 ± 4.7

(La)	F10	Maltodextrin and starch (1:1)	8.8 ± 0.24	9.5 ± 0.12	65.8 ± 5.1
	F11	Maltodextrin and starch (1:2)	9.5 ± 0.26	8.9 ± 0.15	68.7 ± 2.8
	F12	**Maltodextrin and starch (2:1)**	**9.8 ± 0.04**	**9.5 ± 0.14**	**70.5 ± 8.1**

a: Survival is represented as the mean of the log cfu/ml, n=3; b: Yield represented as the mean of percentages of the powder obtained, ± the standard error of the mean.; E*- Encapsulation method (spray drying) (p<0.05)

A decline in shelf life or no viability was observed in case of all LAB after 8 months when formulations are kept at 37 ^{o}C (Fig. 5.11).




Shelf life of encapsulated LAB strain B2
b)
(% viability) cfu g-1
100
80
60
40
20
0
2
4
6
8
10
12
Shelf life in month
L & S (2:1)
M &S (1:1)
M &S (1:2)
M &S (2:1)
L & S* (2:1)
M & S* (1:1)
M & S* (1:2)
M & S* (2:1)

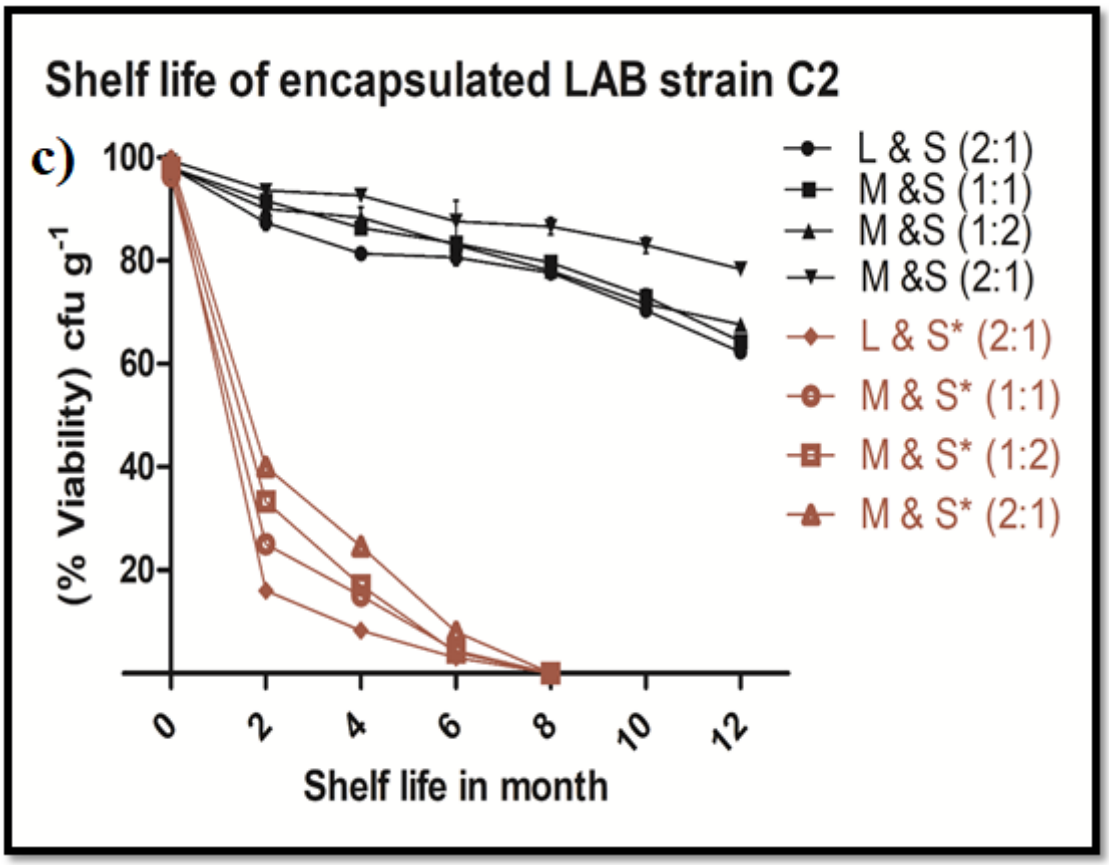

Shelf life of encapsulated LAB strain C2
c)
(% Viability) cfu g-1
100
80
60
40
20
0
2
4
6
8
10
12
Shelf life in month
L & S (2:1)
M &S (1:1)
M &S (1:2)
M &S (2:1)
L & S* (2:1)
M & S* (1:1)
M & S* (1:2)
M & S* (2:1)

Fig. 5.11. Shelf life of encapsulated LAB strain (a) A2, (b) B2, (c) C2 with different viability parameters including Lactose and starch (L & S- 2:1) as ●, Maltodextrin and starch (M & S- 1:1) as ■. Maltodextrin and starch (M & S- 1:2) as ▲, Maltodextrin and starch (M & S- 2:1) as ▼, at 4 ^{o}C and Lactose and starch (L & S- 2:1) as ●, Maltodextrin and starch (M & S- 1:1) as ■. Maltodextrin and starch (M & S- 1:2) as ▲, Maltodextrin and starch (M & S- 2:1) at ▼37 ^{o}C, Values presented are the means and standard deviations from three replicate. Where strains A, B, and C are as *Lactobacillus plantarum, Lactobacillus rhamnosus,* and *Lactobacillus acidophilus* respectively.

5.4.4 Lyophilization of LAB using excipients and their survival studies after encapsulation

The LAB strains of bacteria were analyzed for resistance during the lyophilization using the different lyoprotective excipients. The LAB strain Lr shows cfu/ml values higher in 10% maltodextrin + 5% sucrose and 10% lactose + 5% sucrose i.e. 9.4 ± 0.22, 8.9 ± 0.15 before and 9.1 ± 0.18, 8.6 ± 0.14 after encapsulation. Whereas, in the case of Lp the cfu/ml values found are 9.5 ± 0.05, 9.1 ± 0.21 before and 9.1 ±0.16, 8.4 ± 0.21 after lyophilisation in case of excipients ratio i.e. 10% maltodextrin + 5% sucrose and 10% lactose + 5% sucrose (Table. 5.3). Thus, for ease in understanding the role of lyoprotective excipient, the cfu/g values found in case of skim milk was kept as control with values 7.2 ± 0.14, 7.2 ± 0.24 before and 6.0 ± 21, 6.2 ± 0.16 after lyophilisation in case of Lr and Lp respectively. The values noted for the 10% sucrose as an excipient are found with least cfu/g count with 8.2 ± 0.24, 8.1 ± 0.14 before and 7.4 ± 0.22, 6.5 ± 0.17 values after lyophilisation for Lr and Lp respectively (Table. 5.3). According to Abd-Talib et al.,[1] a different excipient such as gelatin and maltodextrin plays an important role in cells survival rate. In this study, they showed that the reduced cell viability from 3.25×10^7 cfu/ml to 2.15×10^7 cfu/ml during his two-week study model using the different excipients profiling [1]. They also described that the viability of the LAB depends on the excipients used for drying technique. Thus, the same kind of results

is observed in the lyophilisation process showing the importance of different lyoprotective and its effects on viability count.

Table 5.3. Lyophilisation of LAB with the excipients and its cfu/ml count.

Formulation	LAB	Excipient ratio	log cfu/ml (*X10^9)[a]	
			Before L	After L
F13	*Lr*	Skim milk	7.2 ± 0.14	6.0 ± 0.21
F14		10% sucrose	8.2 ± 0.24	7.4 ± 0.22
F15		10% Maltodextrin	9.1 ± 0.36	8.9 ± 0.27
F16		10% Lactose	8.8 ± 0.17	8.2 ± 0.13
F17		**10% Maltodextrin and 5% sucrose**	**9.4 ± 0.22**	**9.1 ± 0.18**
F18		10% Lactose and 5% sucrose	8.9 ± 0.15	8.6 ± 0.14
F19	*Lp*	Skim milk	7.2 ± 0.24	6.2 ± 0.16
F20		10% sucrose	8.1 ± 0.22	6.5 ± 0.17
F21		10% Maltodextrin	9.2 ± 0.27	8.4 ± 0.12
F22		10% Lactose	8.6 ± 0.34	8.1 ± 0.18
F23		**10% Maltodextrin and 5% sucrose**	**9.5 ± 0.05**	**9.1 ± 0.16**

F24		10% Lactose and 5% sucrose	9.1 ± 0.21	8.4 ± 0.21
F25	*La*	Skim milk	7.1 ± 0.14	6.1 ± 0.14
F26		10% sucrose	8.1 ± 0.28	6.3 ± 0.14
F27		10% Maltodextrin	9.2 ± 0.29	8.1 ± 0.13
F28		10% Lactose	8.6 ± 0.31	7.5 ± 0.21
F29		**10% Maltodextrin and 5% sucrose**	**9.4 ± 0.15**	**9.1 ± 0.11**
F30		10% Lactose and 5% sucrose	9.1 ± 0.28	8.1 ± 0.27

a: Survival is described as the mean of the log cfu/ml, n=3; ± the standard error of the mean.; L*- Lyophilisation method (p<0.05)

5.4.5 Viability studies of the lyophilized formulations during the storage

Comparative studies were carried out in the case of LAB strains for long-term stability testing. The lyophilized products were screened for a period of 12 months at two different extreme conditions i.e. at 4 ^{o}C and at 37 ^{o}C. In a case of all strains, significantly higher survival (p<0.05) rate was found at 4 ^{o}C as compared to 37 ^{o}C. During the study skim milk lyophilized product was considered as control. This control group was compared with the rest other lyoprotective excipient combination groups and viability is calculated as per equation 5.1. In case of Lr group at 4 ^{o}C, 10% maltodextrin + 5% sucrose group shows significant higher survival rate i.e. Vsf value of 7.8 (p<0.05) as compared to control group after 12 months studies [fig. 5.12 (A)]. While in the case of Lp group at 4 ^{o}C, 10%

maltodextrin + 5% sucrose group shows highly significant survival (p<0.01) as compared to the control group with the Vsf value around 7.2 [fig. 5.13 (A)]. Similarly, in the case of La group at 4 ^{o}C, 10% maltodextrin + 5% sucrose group shows highly significant survival (p<0.01) as compared to the control group with the Vsf value around 5.2 after 12 months studies [fig. 5.14 (A)]. On the contrary, other excipients group in case of all strains shows comparatively less survival rate after 12 months studies at 4 ^{o}C as compared to 10% maltodextrin + 5% sucrose group.

When the same Vsf study was determined at 37 ^{o}C temperature, it was found that no survival count was found after 6 months of tenure analyzed by serial dilution technique. In case of Lp group at 37 ^{o}C temperature shelf life study, 10% maltodextrin + 5% sucrose group shows highest significant survival (p<0.01) count as compared to control group with Vsf value 3.1 after 6 months study as seen in fig. 5.13 (B). The similar type of study, when carried out in case of Lr at 37 ^{o}C significantly variable results, was obtained (p<0.05) for 6 months study. In case of Lr at 37 ^{o}C using 10% maltodextrin + 5% sucrose group, shows significantly higher (p<0.01) Vsf value i.e. 3.0, after 6 months long-term study as compared to control group as seen in fig. 5.12 (B). Similarly, La at 37 ^{o}C using 10% maltodextrin + 5% sucrose group, shows significantly higher (p<0.01) Vsf value i.e. 2.9, after 6 months long-term study as compared to control group as seen in fig. 5.14 (B). On contrary, other excipients group in case of all strains shows comparatively less survival rate after 6 months studies at 37 ^{o}C as compared to 10% maltodextrin + 5% sucrose group.

T. Ozcan et al. described that in fermented beverages of *L. rhamnosus* in apple and blueberry, the growth of probiotic bacteria is influenced by the physicochemical properties of media along and storage condition at refrigeration [4]. They showed the same results by the short term refrigeration studies based on viability and growth proportion index (GPI) of the LAB.

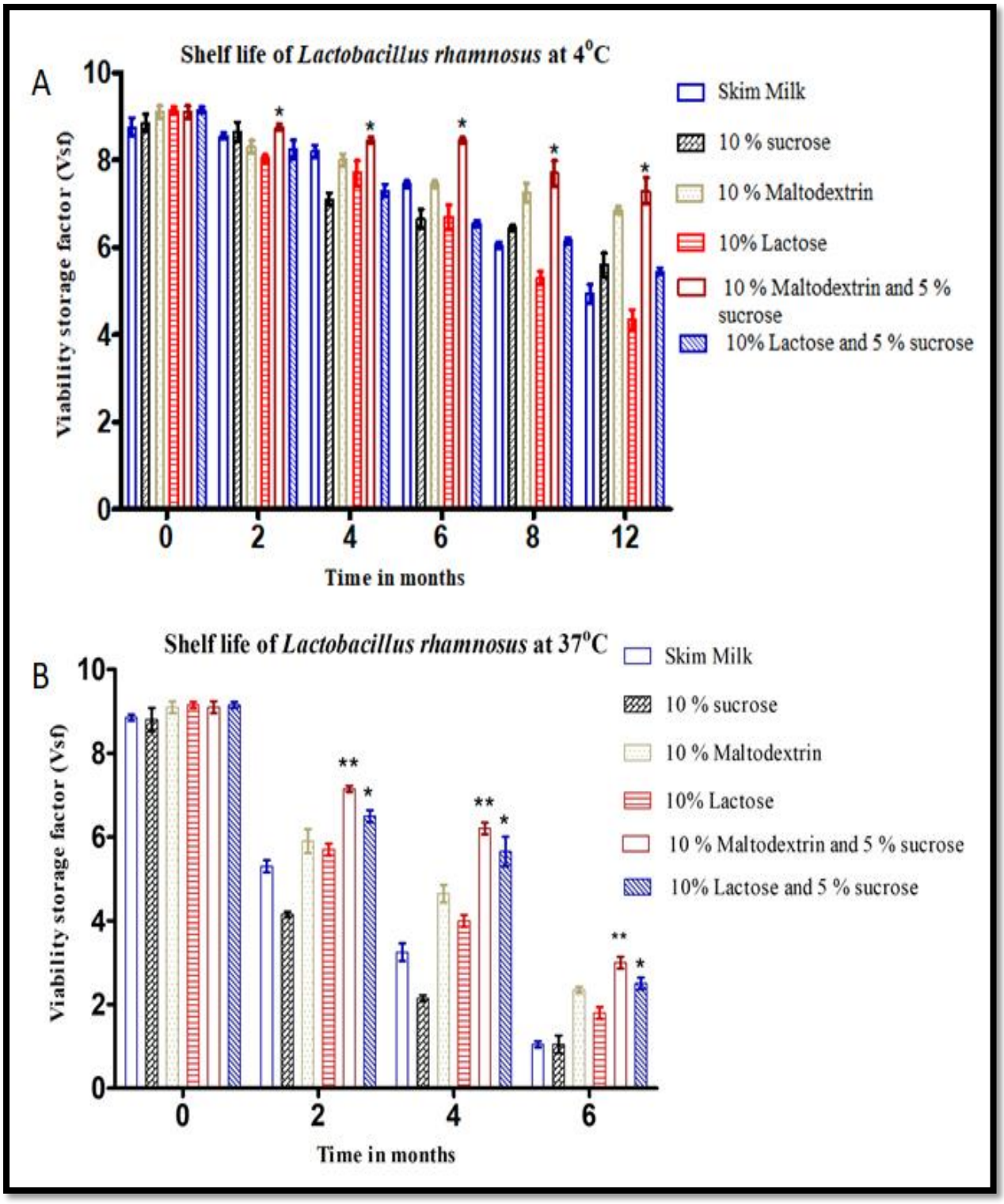


Fig.5.12. Shelf life determination of *Lactobacillus rhamnosus* at different temperature

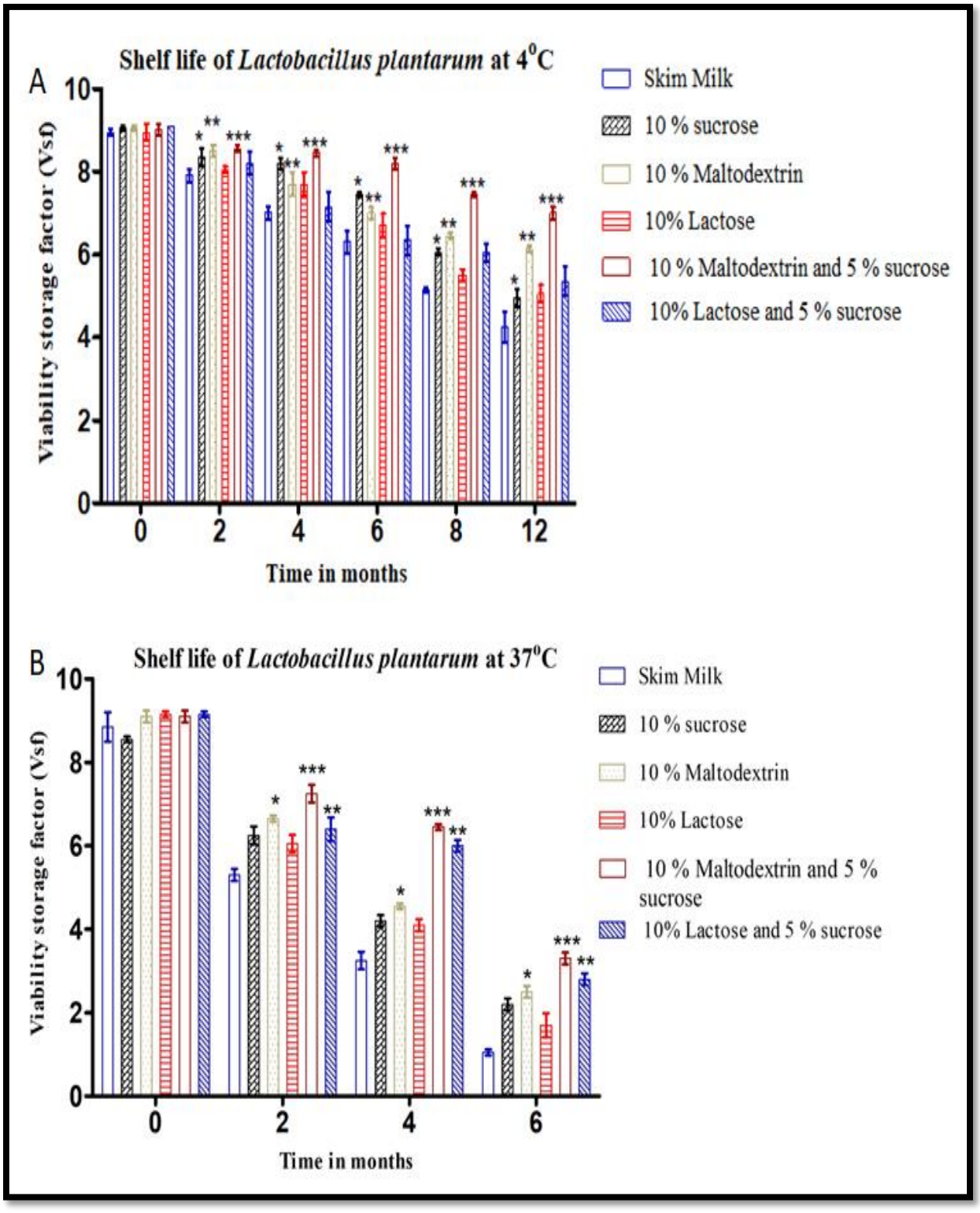


Fig.5.13. Shelf life determination of *Lactobacillus plantarum* at different temperature

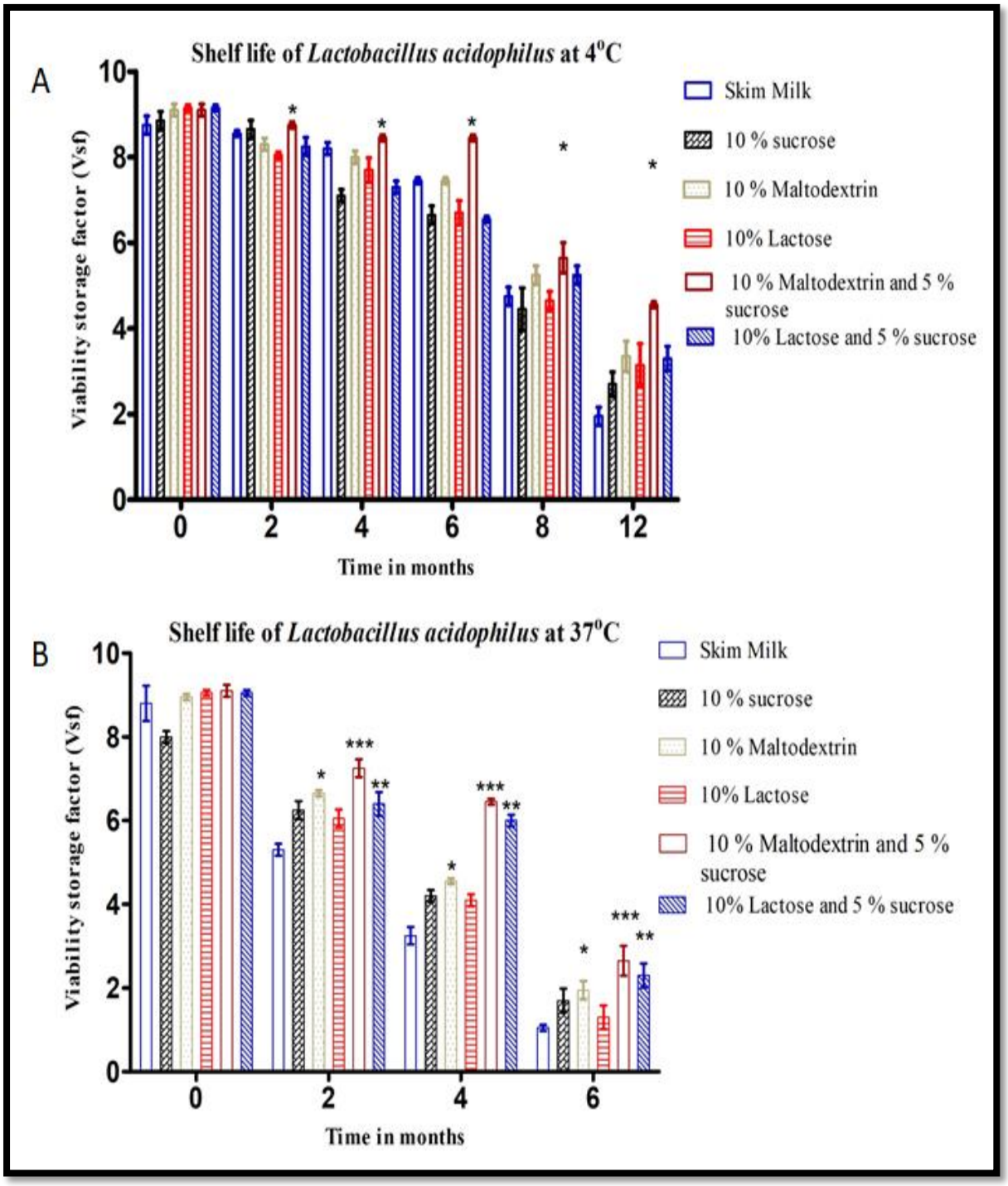


Fig.5.14. Shelf life determination of *Lactobacillus acidophilus* at different temperature

5.4.6 Characterization features of spray dried granules of Lactobacillus

Optimized formula samples were firstly evaluated for sufficient flow properties using various characteristic parameters such as bulk density, tapped density, Hauser's ratio, and compressibility index.

a) Bulk density and tapped density

10.00 g of the LAB granules are taken and transferred into 50 ml cylinder for measuring both tapped and untapped volume. The volume found after tapping of cylinders at 50, 75 and 125 drops are 46, 44 and 42 ml respectively. Calculated bulk density (equation 5.2) and tap density (equation 5.3) are found as follows (Table 5.4);

Table. 5.4. Bulk and Tapped density of granule formulations

Parameter	Formulation observations		
	F4	F8	F12
Bulk density (ρ_{bulk}) (g/ml)	0.20	0.21	0.19
Tapped density (ρ_{Tap}) (g/ml)	0.23	0.25	0.21

b) Compressibility index

The compressibility of material is estimated from the tap density and bulk density measurements (equation 5.4). Compressibility Index obtained are of value 13.04, 16.00, 9.52 for optimised formulations F4, F8 and F12, respectively.

c) Hausner ratio

The value of the Hausner ratio obtained is as follows (equation 5.5)

Table. 5.5. Hausner ratio of granule formulations

Parameter	Formulation observations		
	F4	F8	F12
Hausne'r ratio	1.15	1.19	1.10

Table. 5.6. The standard scale of flow-ability

% Compressibility index	Flow characteristics	Hausner ratio
≤ 10	Excellent	1.00 – 1.11
11 – 15	Good	1.12 – 1.18
16 – 20	Fair	1.19 -1.25
21 – 25	Passable	1.26 – 1.34
26 – 31	Poor	1.35 – 1.45
32 – 37	Very poor	1.46 – 1.59
≥ 38	Very, very poor	> 1.60

The standard scale of flow-ability is compared with the obtained results of Hausner ratio [44]. From the above results of the compressibility index and Hausner ratio value, it is found that the material exhibits good flow properties as per table 5.6.

d) Angle of repose

10 g powder was taken and then passed through the funnel. The height of the cone and average diameters (D) of the circle formed at the base of the cone-like pile are measured. The radius (r=D/2) of the circle formed by the cone-like pile is measured. The calculation is done as enumerated below as equation 5.6.

Table 5.7. The angle of repose for the optimized spray-dried Lactobacillus granules

Parameter	Formulation observations		
	F4	F8	F12
Angle of repose (range)	++	++	+++
Flow properties	Good	Good	Fair

Table 5.8. The standard chart of flow property and the corresponding angle of repose

Flow Property	The angle of Repose (degrees)	Range
Excellent	25 – 30	+
Good	31 – 35	++
Fair: aid not needed	36 – 40	+++
Passable: may hang up	41 – 45	++++
Poor: must agitate or	46 – 55	+++++
Very poor	56 – 65	++++++
Very, very poor	> 66	+++++++

The standard chart of flow property and the corresponding angle of repose is compared with the obtained results of the angle of repose for the optimized spray-dried Lactobacillus granules [44]. From the above results of the angle of repose, it is found that the *L. plantarum* and *L. rhamnosus* granules exhibit good flow properties as per Table 5.8 compared to *L. acidophilus*.

From the bulk and tap density study, compressibility index, Hausner ratio, and angle of repose studies it is concluded that flow analysis of all optimised formulations i.e. F4, F8 and F12 are having nearly same good flow properties.

e) Loss on drying

The instrument used for this purpose was Halogen Moisture Analyzer. The percentage loss on drying is found 2.1, 2.4 and 2.7 for formulations F4, F8 and F12 respectively on auto mode.

5.4.7 Solubility and hygroscopicity of LAB formulations

The solubility percentage of all the LAB samples ranged between 58.27% and 67.25%. Solubility is found high in case of Lp with the formulation F4 and decreased in formulation F12 (La) in case of spray dried encapsulation technique. Similarly, the solubility was found high in case of Lp with the formulation F17 and decreased in formulation F29 (La) in case of lyophilisation encapsulation technique. It shows that the higher molecular weight of the coating material influences the solubility of the spray dried and lyophilised powders.

Table 5.9. The solubility of Lactobacillus strains in water

Formulations	Isolates	Solubility (%)
F4	Lp	67.25 ± 0.18
F8	Lr	65.75 ± 0.41
F12	La	59.27 ± 0.17

F17	Lp	60.12 ± 0.22
F23	Lr	61.75 ± 0.21
F29	La	58.27 ± 0.21

n=3; ± the standard error of the mean

Table 5.10. Hygroscopicity of Lactobacillus strains in water

Formulation	Isolates	Hygroscopicity (g/100 g)
F4	Lp	13.92 ± 0.19
F8	Lr	15.21 ± 0.21
F12	La	23.22 ± 0.32
F17	Lp	11.72 ± 0.21
F23	Lr	14.27 ± 0.41
F29	La	21.16 ± 0.12

n=3; ± the standard error of the mean

Hygroscopicity was found low in case of Lp with the formulation F4 and increased significantly in (La) formulation F12 in case of spray dried granules. Similarly, the hygroscopicity was found low in case of Lp with the formulation F17 and increased significantly in formulation F29 (La) in case of lyophilised granules, may be due to its porous nature.

5.4.8 Titratable acidity (%) of LAB formulations

All the LAB powders show a titratable acidity value from 0.30% - 0.33%.

Table 5.11. Titratable acidity (%) of lactobacillus strains

Formulation	Isolates	Titratable acidity (%)
F4	Lp	0.30 ± 0.02
F8	Lr	0.32 ± 0.04
F12	La	0.29 ± 0.05
F17	Lp	0.26 ± 0.03
F23	Lr	0.29 ± 0.04
F29	La	0.27 ± 0.02

n=3; ± the standard error of the mean

Titratable acidity is found low in cases of the F4 and F12 formulations by a spray dried encapsulation technique. Similarly, the titratable acidity is found low in cases of Lp with the formulation F17 and La formulation F29 by lyophilisation encapsulation process. The lowering in the titratable acidity may be due to the porous nature of the granules.

5.4.9 SEM analysis of LAB formulations

The optimised formulations of LAB are evaluated by the SEM analysis.

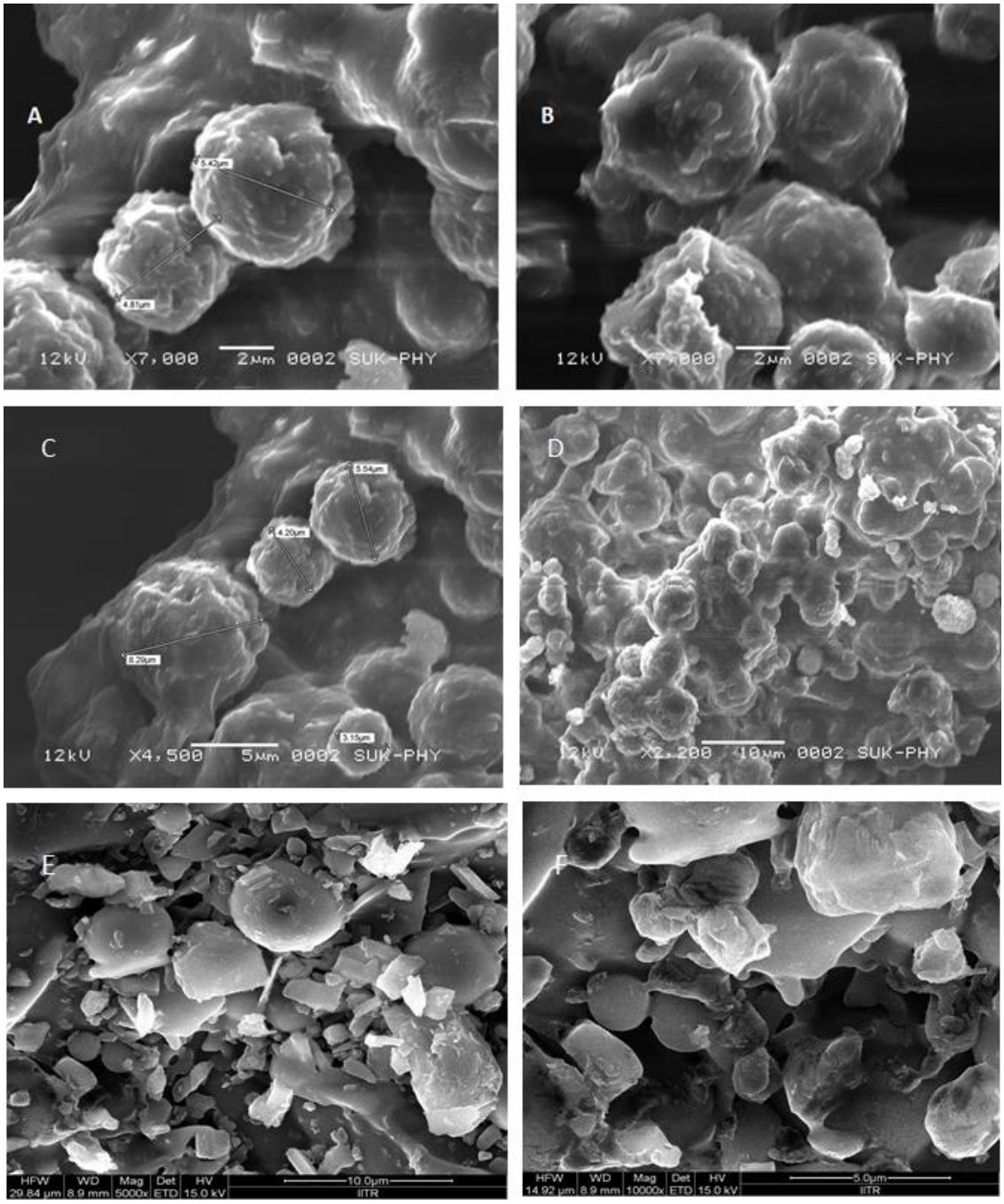


Fig. 5.15. Scanning electron microscopic images of A) *Lactobacillus plantarum* (F4), B) *Lactobacillus rhamnosus* (F8) and C) *Lactobacillus acidophilus* (F12) of spray dried granules with its dimensions; D) *Lactobacillus plantarum* (F17), E)

Lactobacillus rhamnosus (F23) and F) *Lactobacillus acidophilus* (F29) of lyophilized powder; Magnification range from X2,200 – X7,000

In this analysis, the particle size observed in the case of spray-dried formulations are in the size of the 4.5 to 5.8 µm and showing uniform particle size distribution. While in cases of the lyophilized formulations the particle size are found in the range of 2.4 to 10.5 µm and not showing uniform particle size distribution, due to high hygroscopicity as compared to spray dried granules.

5.5. Conclusions

This chapter provides information that LAB inoculations in the milk of cow, buffalo, goat, and sheep milk show significant growth with an optimal high growth rate in buffalo milk. Further, Mehsana milk is found more suitable for the flourishing of the LAB due to the high cfu growth rate of LAB in it as compared to other buffalo milk. The studies showed that the LAB culture can be formulated as dry granules by spray dry and lyophilisation process. The viability studies shows that the shelf life of the formulations not only depends upon the combination of excipients for drying but also upon the temperature of storage. It is also found that the type of excipients used and method of encapsulation used, affect the granules flow properties and particle sizes. The shelf life study shows that the viability of the granules increases by storage at 4 ^{o}C temperature as compared to 37 ^{o}C in case of both drying methods. It is also concluded that dry granules produced by the spray drying method are cost-effective and produced in less time as compared to the lyophilisation.

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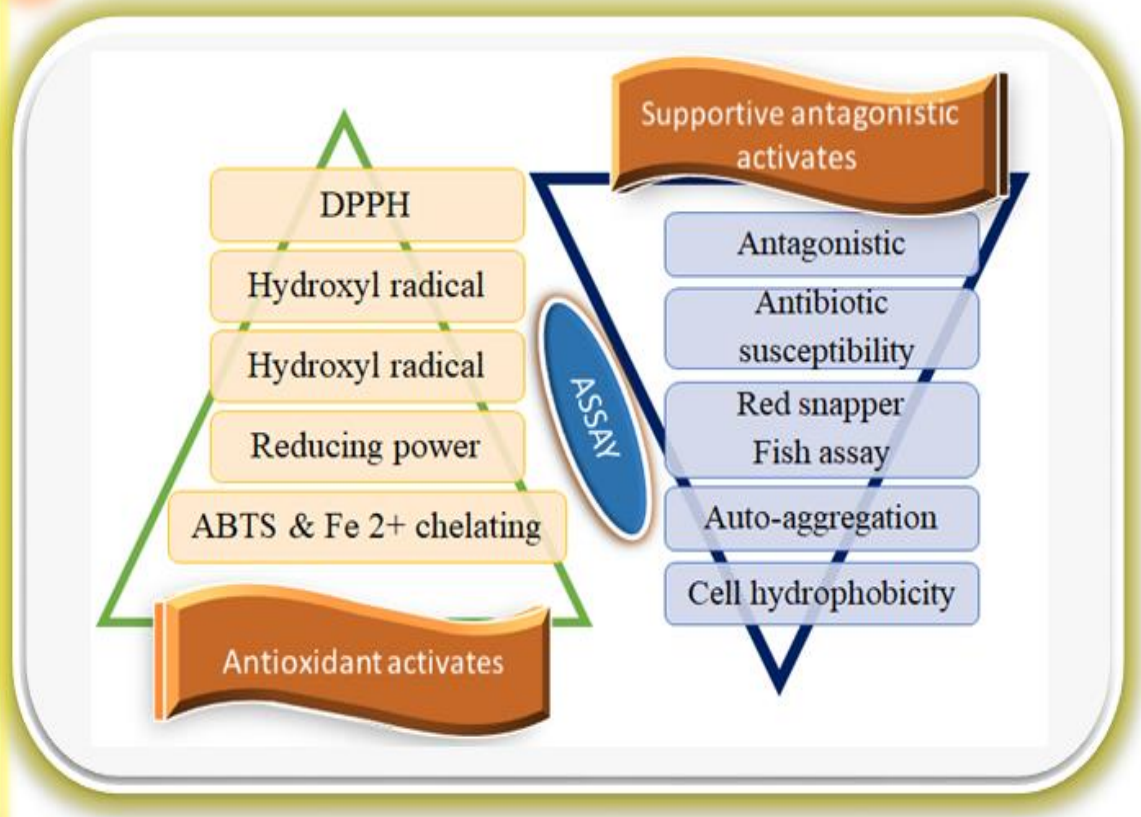
Chapter 6

Bacteria keeps us from heaven and puts us there- Martin Fischer

In-vitro antioxidant and supportive antagonistic activities of Lactobacillus



6.1 Introduction

Lactobacillus genera used as a functional food like curd and butter are found to treat minor gastrointestinal related disorders. These LAB from milk products and gastrointestinal tract are found as more effective prophylactic agents [1]. LAB prevents the dysbiosis and kills mostly the pathogenic microbes. *Lactobacillus plantarum* and *Lactobacillus rhamnosus* are considered as the most important lactic acid bacteria conferring health benefits [2]. These LAB are made available as various formulations in the market. Many probiotics formulations are available as a granule and tablet formulations [3]. The probiotics formulations available in the market are having a short shelf life. The shelf life issue if solved these dosage form acts as a good neutraceutical mediator [4]. The dose of 10^8 cfu counts is enough to show the health benefits [5]. Fall in cfu count results in zero pharmacological activities [6]. Spray drying yogurt is used as the best source of *Lactobacillus* in dairy as starter cultures [7]. Lyophilized and spray dry probiotic cultures, confer health benefits to host [8]. These powder formulations once rehydrated can be used as functional food [9].

Probiotic properties such as cell adhesion, auto-aggregation, and hydrophobicity are found vital, to fight against pathogens. Cell adhesion of LAB is studied by many researchers using the fish intestine mucus model. Physiochemical abilities of cell surface are found vital in cell adhesion to the intestinal mucosa by means of hydrophobicity [10]. *In-vitro* studies in LAB are investigated and are found as preliminary potential mediators to bind to the host cells [11]. Many investigators tried to find out the relationships between hydrophobicity and cell adhesion but failed to show the correlation between them [12].

The present study deals with the investigations of antagonistic activities of Lp and Lr isolated from sheep milk. Further, the antibiotic susceptibility test using different generation of antibiotics is demonstrated on Lp, Lr, and La. The synergistic role of cell hydrophobicity along with the cell adhesion studies supporting the antioxidant activity was investigated in the following study. La is used as the reference standard during the study.

6.2 Experimental

6.2.1 Cell preparation for *in-vitro* antioxidant activity and supportive antagonistic activities

The LAB isolated from sheep milk using selective MRS, media were incubation for 37 ^{o}C, at 24 h in an anaerobic jar. After incubation, the selected colonies were transferred into MRS broth media at 37 ^{o}C with an adjusted pH of 6.5 for 18 h. The freshly prepared culture of LAB with a cell count of 10^8 cfu/ml, was centrifuged at 3000 rpm for 5 min at 4 ^{o}C. The supernatant is separated and pellet formed was added to 0.5 ml of PBS and was used as an intact cell for further experiments. Similarly, the obtained supernatant was passed through the syringe filter of 0.2 µm filter and was used for *in-vitro* supportive antagonistic activities.

Similarly, the lysate of LAB was prepared by the method described by Kamal et al., [13]. Briefly, 100.0 ml of LAB with a cell count of 10^8 cfu/ml were centrifuged at 12,000 g, 5 min at 4 ^{o}C. The cells were washed with PBS with the addition of 100 µl lysing buffer [14]. Finally, the cells were disrupted by the ultra-sonication in the ice bath for about 60 s. The lysate obtained was passed through the syringe filter of 0.2 µm filter and was used for further experimentation.

6.2.2 Antioxidant activities of Lactobacillus

A) DPPH assay of LAB

The scavenging capacity of the LAB strains was investigated by DPPH assay according to the method described by Rahman et al., with some modification [15]. Briefly, 1 ml of lysate and intact cells were added separately to 2.1 ml of ethanolic DPPH radical solution (0.01 mM). This mixture was incubated with the vigorous stirring in the dark for 30 min. Deionized water and DPPH solution were used as the controls. The absorbance of the resulting solution was measured in triplicate at 517 nm after centrifugation at 7200 g for 10 min. The scavenging ability was determined by equation 6.1. *L. acidophilus* was used as the reference standard for this assay.

Scavenging ability (%) = $(A_0-A_t)/A_0$ x 100 (6.1)

Where, A_0 is the absorbance of the control, and A_t is the absorbance of the cell free extract.

B) Scavenging of hydroxyl radicals of LAB

The scavenging of hydroxyl radicals by LAB strains was measured according to Ding et al., with slight modification [16]. Briefly, the reaction mixture was prepared using 0.8 ml of O-phenanthroline (0.12%, w/v), 0.9 ml of 3.0 mM FeSO4, 1.5 ml of 30 mM H_2O_2, and 1 ml of PBS. 1 ml of lysate and intact cells solutions were added separately to the reaction mixture. The formed mixtures were further incubated at 37 ^oC for 2 h in a water bath. The percentage of free radical-scavenging was determined at an absorbance of 540 nm. The scavenging ability was calculated as per equation 6.2. *L. acidophilus* was used as the reference standard for this assay.

Scavenging ability (%) = $[(A_a-A_b)/ (A_a-A_0)]$ x 100 (6.2)

Where A_0 is the control group absorbance in the absence of the H_2O_2 and sample, A_a is the absorbance without actual sample but in the presence of H_2O_2, A_b is the absorbance of the sample with H_2O_2.

C) Scavenging of the superoxide radical of LAB

The superoxide radical scavenging activity by LAB strains was assessed by the method of Ding et al., with a slight modification [16]. The reaction mixture was prepared using the Tris-HCl butter (2.7 ml, pH 7.9, 0.05 M) and pyrogallic acid (0.2 ml, 0.05 M). The reaction mixture was added to 0.1 ml of lysate and intact cells solutions separately. The reaction mixtures were kept at 25 ^oC for 4 min in the dark. The reaction was terminated by addition of 0.8 ml (8 M HCl). Absorbance was measured at 300 nm and the superoxide radical-scavenging activity (%) was calculated as per equation 6.3. *L. acidophilus* was used as the reference standard for this assay.

Scavenging rate (%) = [1-A_{300nm} sample/A_{300nm} blank] x 100% (6.3)

Where A_{300nm} sample is absorbance in the presence of LAB as lysate and intact cell, A_{300nm} blank is absorbance in absence of LAB

D) Reducing power assay of LAB

Reducing power activity by LAB strains was determined according to the method described by the Ding et al., with slight modification [16]. The reaction mixture was prepared by using the 0.6 ml of 1% potassium ferricyanide, 1 ml of 0.1 M PBS. The reaction mixture was added to 0.5 ml of lysate and intact cells solutions separately. Further, the mixture kept in a water bath for 10 min at 60 ^{o}C. The mixture was cooled with the addition of 1 ml of 20% trichloroacetic acid. The final mixture was centrifuged at 5500 g, for 8 min at 4 ^{o}C. 1 ml of the reaction mixture was removed and 0.5 ml of 0.2% ferric chloride was added to it. Finally, the absorbance was measured at 690 nm. The reducing power was determined as directly proportional to reducing the power of the sample. *L. acidophilus* was used as the reference standard for this assay.

E) ABTS assay of LAB

The scavenging of 2,2'-azino-bis-(3-ethylbenzothiazoline-.6-sulfonic) acid (ABTS) radical by LAB strains was determined according to the method described at Helmy et. al., with slight modifications [17]. The reaction mixture was prepared using combination of 6 mM ABTS with 2.5 mM potassium persulfate (1:1 v/v) solution. The reaction mixture was added to 0.5 ml of lysate and intact cells solutions separately. The formed mixture was kept for 14 h to stabilise the reaction; monitored till contact absorbance was noted. The stabilised reaction mixture was added with ethanol until the constant absorbance of 0.690 ± 0.08, observed at 736 nm. The photometric assay was evaluated at 734 nm after 60 s of the reaction. The antioxidative activity was evaluated by the equation 6.4. *L. acidophilus* was used as the reference standard for this assay.

$$E= [(Ac-At)/Ac] \times 100 \quad (6.4)$$

Where At is the absorbance of LAB and Ac is the absorbance of ABTS•+

F) Fe2+ chelating assay of LAB

The Fe2+ chelating ability of LAB was estimated using the method of Tang et al. [18] with slight modifications. The reaction mixture was prepared using 1 ml of NaOH (0.3 M), 0.2 ml of $FeSO_4$ (2 g/l) and 0.2 ml of ascorbic acid (5 g/l). The reaction mixture was added to 0.5 ml of lysate and intact cells solutions separately. The mixtures were kept in a water bath for 15 min at 37 °C. 0.3 ml (9%) of trichloroacetic acid was added to final reaction mixtures with the centrifugation of 7200 rpm for 10 min. The 1.5 ml supernatant was obtained from the final mixture which was added to 10% phenanthroline (1 g/l). Finally, the absorbance was observed after 10 min at 500 nm. *L. acidophilus* was used as the reference standard for this assay.

6.2.3 *In-vitro* supportive antagonistic studies of Lactobacillus

Antagonistic activities are vital for any probiotics species to contribute to host positive immune-activity. This is one of the important parameters that define the probiotic potential of any microbes. These antagonistic attributes do not come in alone but consist of a cascade of supportive events called as supportive antagonistic activities. This supportive events consist of auto-aggregation, hydrophobicity and antibiotic's susceptibility.

A) Antagonistic activity of isolates

Antimicrobial activity of isolates against pathogenic strains was assessed using agar disc diffusion method [19]. Test microorganisms used were gram-positive *P. vulgaris, E. coli, P. aeruginosa, S. typhi* and gram-negative microbes *B. subtilis, B. cereus, E. faecalis*. The pathogen (100 µl) was added to soft agar, mixed and overlaid on Muller Hinton Agar (MHA) which was purchased from Hi-Media, USA. A 100 µl of intact cells as a whole organism, its supernatant and lysates were separately plated using a disc on the agar plates. Plates were allowed to dry and

incubated at 37 °C for 34 h. Ciprofloxacin 25 µg/ml was used as the reference standard for the study.

B) Antibiotic susceptibility

The antibiotic susceptibility test of LAB was carried out using the different antibiotic disc on MHA plates [20]. The plates of MHA were prepared and solidified at room temperature. Freshly prepared 100 µl of intact cells as a whole organism, its supernatant and lysates were separately spread on MHA plates. On the MHA plates, different types of antibiotic discs were placed and were incubated for 48 h at 37 °C. The antibiotic zone scale was used to determine the zone of inhibition due to LAB. The obtained result was evaluated as resistance, moderate susceptibility or as susceptibility. The assessment of antibiotic susceptibility pattern was done using Kanamycin (30 µg), Streptomycin (10 µg), Azithromycin (10 µg), Erythromycin (10 µg), Vancomycin (30 µg), Nalidixic acid (30 µg), Tetracycline (30 µg), Penicillin G (10 µg), Ampicillin (10 µg), Gentamicin (10 µg), Chloramphenicol (30 µg), and Ciprofloxacin (10 µg).

C) Auto-aggregation assay

This assay was performed by incubating Lp, Lr and La strains of different formulations of spray dry (F1 - F12) and lyophilization (F13 - F30) granules in MRS broth at 37 ^{o}C for 24 h. The prepared formulations were incubated in MRS broth for 18 h at 37 ^{o}C. The generated cells of count 10^{7-8} cfu/ml were centrifuged at 3000 g for 15 min [10,20]. The 4 ml of the cell suspension was a vortex for 20 s and 1 ml of the upper layer was used to determine the auto-aggregation activity. The activity was measured after 5 h at 620 nm absorbance.

The auto-aggregation (Aa) was determined and calculated as-

$$Aa = (I\text{-}OD/F\text{-}OD)/ I\text{-}OD \times 100, \quad (6.5)$$

Where, I-OD denotes initial optical density; F-OD denotes final optical density after 5 h.

D) Cell surface hydrophobicity

Bacterial adhesion is the unique property which determines its adhesion to the mucosal surface of the gut. The potential of adherence by microorganisms is determined by its affinity to the surface hydrocarbons. The cultures of different formulations of spray dry (F1 - F12) and lyophilization (F13 - F30) formulations were allowed to grow in MRS broth for 16–18 h at 37 ^{o}C and were centrifuged. The pellets obtained are washed twice with phosphate-urea-magnesium-sulfate buffer. The washed pellets were vortex and adjusted to absorbance 0.7–0.9 at 600 nm. The organic and aqueous phase of cell suspension (3.0 ml) was separated using 1 ml of xylene by incubation at 37 °C for 45 min. 1 ml of the aqueous phase was observed for the absorbance of 610 nm (A_A). Percent of hydrophobicity was calculated using equation 6.6 as described by Lee et al., [21].

Percentage (%) hydrophobicity = $(1-A_A/A_B) \times 100$, (6.6)

Where A_B- blank reading without the sample.

***E) In-vitro* cell adhesion techniques using *Poecilia reticulate* (common guppy)**

The *Poecilia reticulate* also known as common guppy fish were purchased from a local market (Ratnagiri, India). These fishes were strived for 24 h and were scarified. The intestine of the fishes were transferred to the petri dish. The mucus of gut was removed by scrapping mucosal surface [10]. The mucus was then homogenized and centrifuged at 8000 g for 20 min at 4 ^{o}C in order to remove the other cellular debris. Finally, the mucus suspension was sterilized in U.V. light and stored in an aseptic container at -20 ^{o}C. Later, 100 µl of the MRS broth inoculated for 24 h at 37 ^{o}C by different formulations of spray dry (F1 - F12) and lyophilization (F13 - F30) granules were added separately into the 200 µl solution of freshly prepared mucus suspension, previously coated on the microtiter plate wells. Further, the LAB cells were incubated for 1 hr at 37 ^{o}C with the mucus suspension. The incubated suspension was washed thrice with the saline solution to remove the non-adherent cells. The suspension was kept at 60 ^{o}C for 15 min and was stained with

1% crystal violet for 40 min. The excess of stain was removed by saline solution wash and absorbance was taken at 620 nm using microtiter plate reader. Mucus stained without LAB was considered as control. While the final absorbance values were recorded by subtracting the control readings.

6.3 Results and Discussion

6.3.1 Antioxidant activities of Lactobacillus

A) Scavenging activity of Lp, Lr and La by DPPH assay

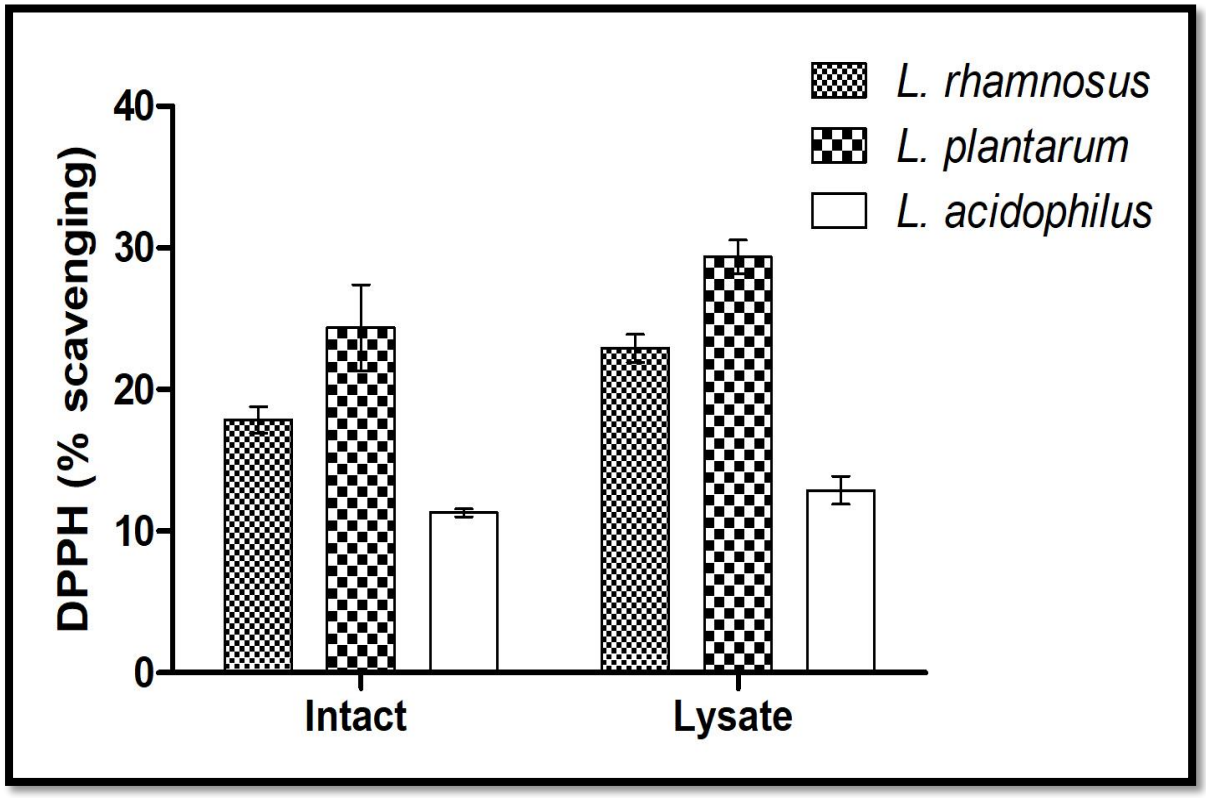


Fig 6.1. Scavenging of DPPH radicals by lactobacillus, ± indicate standard error of means, n=3.

The antioxidant activity determined by the scavenging of DPPH radicals shows that the Lp as lysate with 29 ± 1.4% scavenging activity, significantly greater than intact cell 25 ± 5.2% (Equation 6.1). Similarly, Lr as lysate shows 22 ± 1.8% significantly greater scavenging activity than intact cell 18 ± 2.1%. While in the case of La (reference standard) as the lysate, shows 12 ± 1.8% scavenging activity nearly same as that of intact cell 11 ± 0.7% which is non-significant in nature (Fig. 6.1).

B) Scavenging of hydroxyl radicals by Lp, Lr, and La

The antioxidant activity determined by the scavenging of hydroxyl radicals shows that the Lp as lysate with 79 ± 2.4% scavenging activity, significantly greater than intact cell 70 ± 5.4% (Equation 6.2). Similarly, Lr as lysate shows 71 ± 1.8% significantly greater scavenging activity than intact cell 68 ± 2.1%. While in the case of La (reference standard) as the lysate, shows 24 ± 1.8% scavenging activity nearly the same as that of intact cell 19 ± 3.1% which is non-significant in nature (Fig. 6.2).

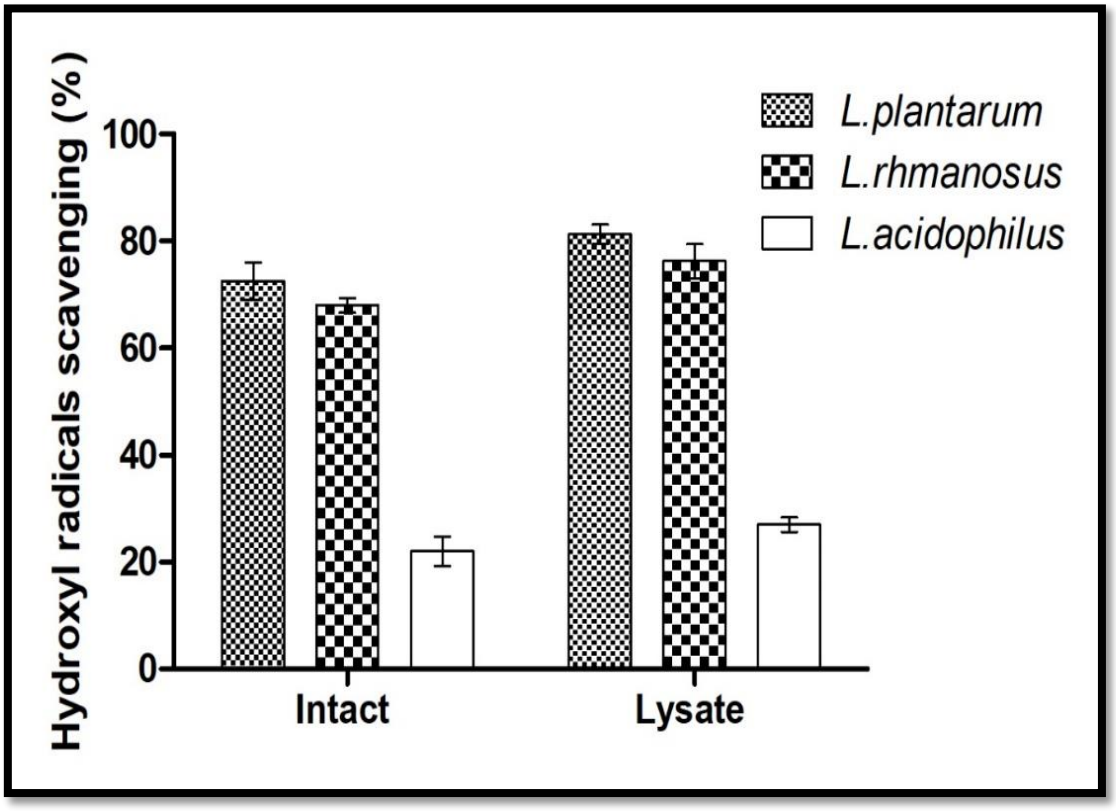


Fig 6.2. Scavenging of hydroxyl radicals by lactobacillus, ± indicate standard error of means, n=3.

C) Scavenging of the superoxide radical by Lp, Lr and La

The antioxidant activity determined by the scavenging of superoxide radical radicals shows that the Lp as lysate with 70 ± 1.4% scavenging activity, significantly greater than intact cell 63 ± 5.4% (Equation 6.3). Similarly, Lr as lysate shows 61 ± 1.1% significantly greater scavenging activity than intact cell 55 ± 2.1%. While in

the case of La (reference standard) as lysate, shows 20 ± 3.8% scavenging activity significantly greater scavenging activity than intact cell 11 ± 4.1% (Fig. 6.3).

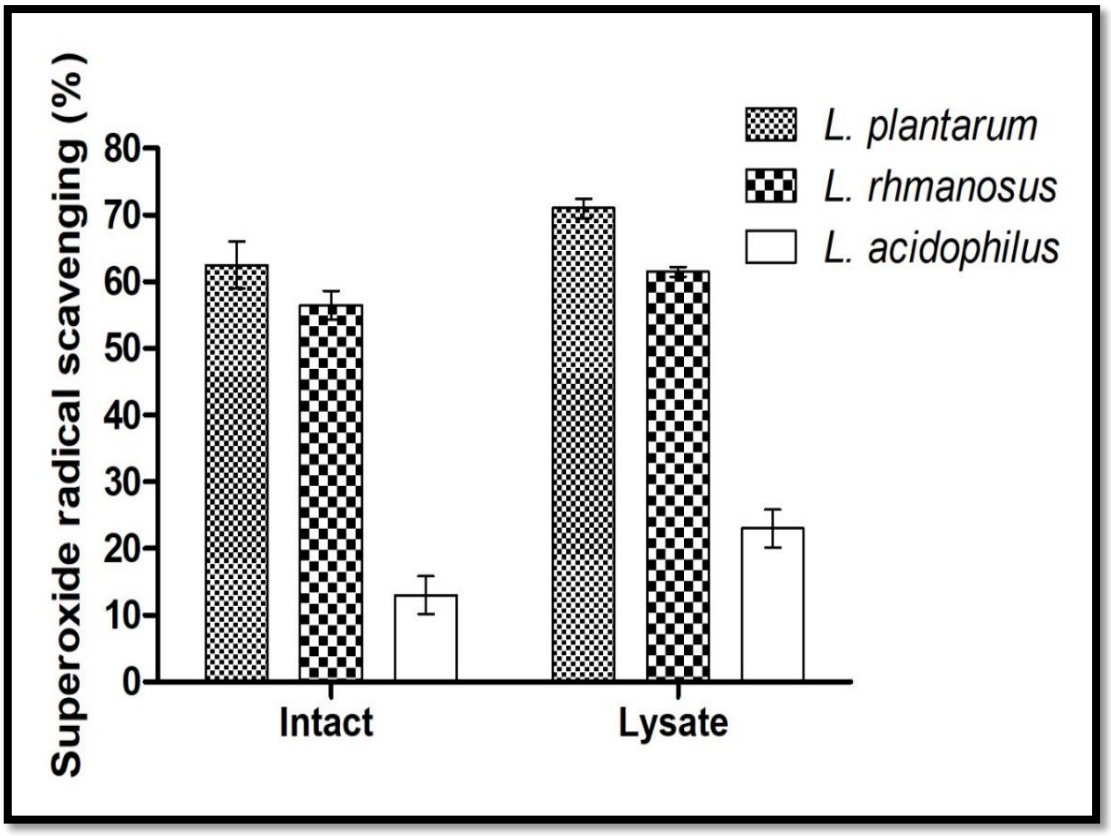


Fig 6.3. Scavenging of superoxide radical radicals by lactobacillus, ± indicate standard error of means, n=3.

D) Reducing power assay by Lp, Lr and La

The antioxidant activity determined by the scavenging activity of reducing power assay shows that the Lp as lysate with 28 ± 1.4% scavenging activity significantly greater than intact cell 20 ± 1.6%. Similarly, Lr as lysate shows 18 ± 2.1% significantly greater scavenging activity than intact cell 16 ± 3.1%. While in the case of La (reference standard) as lysate, shows 10 ± 3.8% scavenging activity significantly greater scavenging activity than intact cell 07 ± 2.2% (Fig. 6.4).

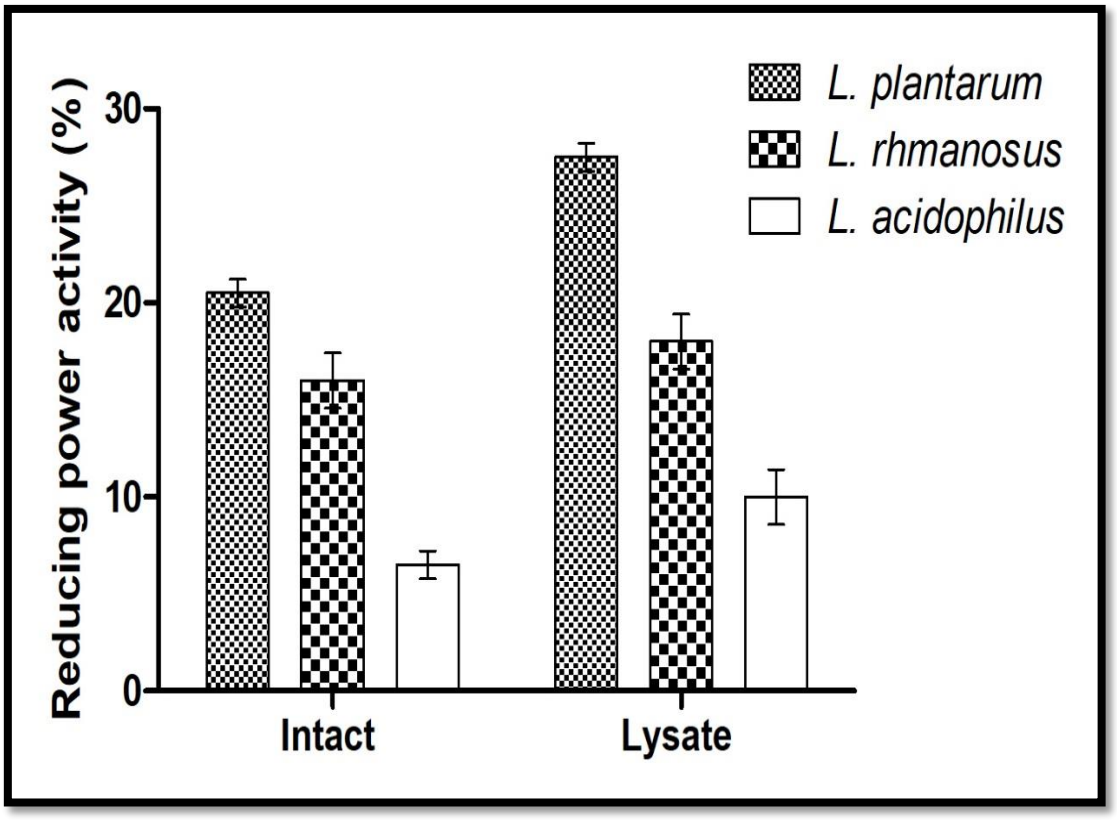


Fig 6.4. Scavenging of reducing power radicals by lactobacillus,

± indicate standard error of means, n=3.

E) ABTS assay by Lp, Lr and La

The antioxidant activity determined by the scavenging activity of ABTS assay shows that the Lp as lysate with 86 ± 1.4% scavenging activity nearly same as intact cell 83 ± 1.6% (Equation 6.4). Similarly, Lr as lysate shows 78 ± 2.1% nearly the same scavenging activity than intact cell 76 ± 1.1%. While in the case of La (reference standard) as lysate, shows 22 ± 1.8% scavenging activity significantly greater scavenging activity than intact cell 17 ± 2.2% (Fig. 6.5).

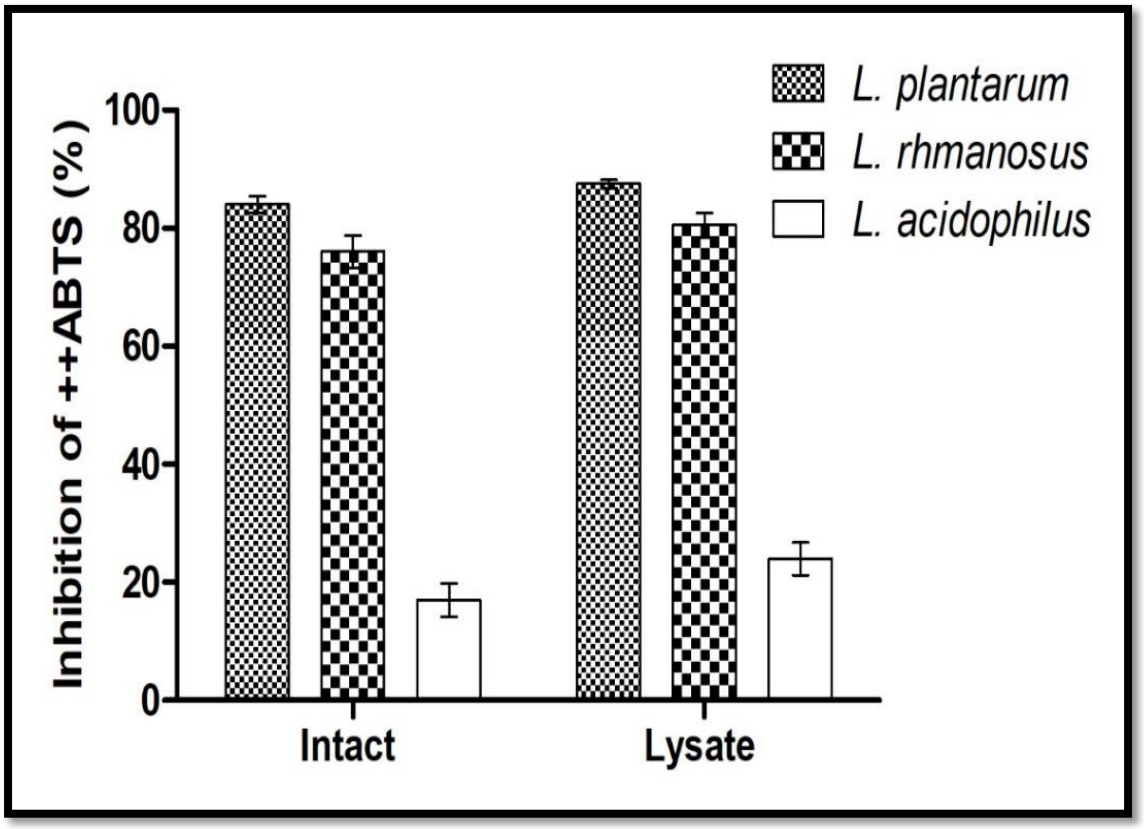


Fig 6.5. Scavenging of ABTS radical by lactobacillus, ± indicate standard error of means, n=3.

F) Fe2+-chelating assay by Lp, Lr and La

The antioxidant activity determined by the scavenging activity of Fe2+-chelating ion assay shows that the Lp as lysate with 45 ± 1.4% scavenging activity significantly greater than intact cell 34 ± 2.6%. Similarly, Lr as lysate shows 32 ± 2.1% significantly greater scavenging activity than intact cell 24 ± 2.2%. While in the case of La (reference standard) as lysate, shows 21 ± 1.8% scavenging activity significantly greater scavenging activity than intact cell 17 ± 3.2% (Fig. 6.6).

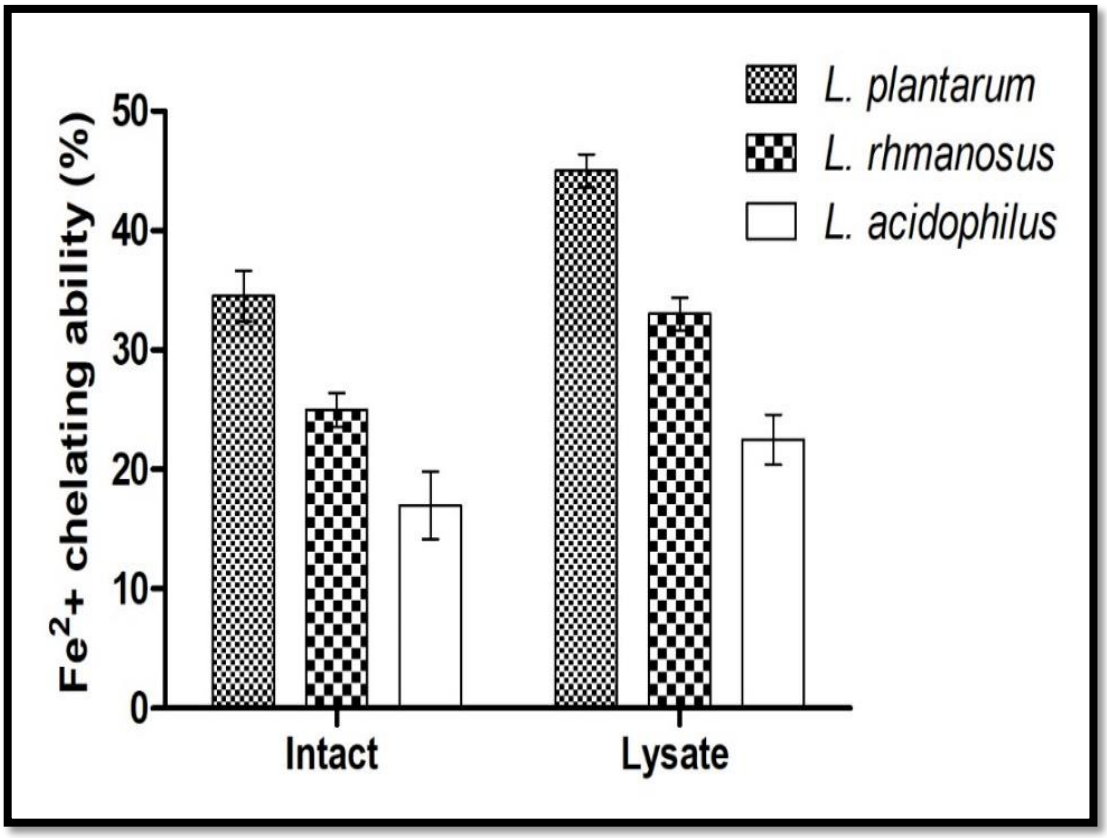


Fig 6.6. Scavenging of Fe2+ chelating ion radical by Lactobacillus,

± indicate standard error of means, n=3.

6.3.2 Evaluation of supportive antagonistic activities of Lactobacillus

A) Antagonistic activity of Lp, Lr and La

LAB as the whole cells, supernatant and lysate are tested for antimicrobial activity against common enteric pathogens (Table 6.1). On the basis of inhibition against all tested Gram-positive and Gram-negative organisms, the maximum zone of inhibition is observed for Lp. Intact Lp compared to its lysate and supernatant shows significant antagonistic activity, even greater than reference standard La. Similarly, the isolate of Lr especially the intact cells shows more inhibitory effect toward tested pathogens as compared to the reference standard. La, is found ineffective in the antagonistic activity against both Gram-positive and Gram-negative pathogenic strains in case of intact cells, supernatant, and in its lysate form.

Table 6.1. Zone of inhibition studies of the Lactobacillus intact cells

Test organism nature		Zone diameter (ZOD) in mm (Mean ± S.D)			
		Intact cell			Ciprofloxacin
		Lp	Lr	La	
Gram positive	*B. subtilis*	+++	+++	-	+++
	B. cereus	+++	++	-	+++
	E. faecalis	+++	++	-	+++
Gram negative	*P. vulgaris*	+++	+++	-	+++
	E. coli	+++	++	-	+++
	P. aeruginosa	+++	++	-	+++
	S. typhi	+++	++	-	+++

ZOD: - no inhibition, + zone of diameter less than 1mm, ++ zone of diameter between 2 to 5 mm, +++ zone of diameter between 5 to 10 mm, ± indicate standard error of means, n=3.

Table 6.2. Zone of inhibition studies of the Lactobacillus supernatant

Test organism nature		Zone diameter (ZOD) in mm (Mean ± S.D)			
		Supernatant			Ciprofloxacin
		Lp	Lr	La	
Gram positive	*B. subtilis*	++	++	-	+++
	B. cereus	+++	++	-	+++
	E. faecalis	++	++	-	+++
Gram negative	*P. vulgaris*	++	++	-	+++
	E. coli	++	++	-	+++
	P. aeruginosa	++	++	-	+++
	S. typhi	++	++	-	+++

ZOD: - no inhibition, + zone of diameter less than 1mm, ++ zone of diameter between 2 to 5 mm, +++ zone of diameter between 5 to 10 mm, ± indicate standard error of means, n=3.

Table 6.3. Zone of inhibition studies of the Lactobacillus lysates

Test organism nature		Zone diameter (ZOD) in mm (Mean ± S.D)			
		Lysate			Ciprofloxacin
		Lp	Lr	La	
Gram positive	*B. subtilis*	++	++	-	+++
	B. cereus	+++	++	-	+++
	E. faecalis	++	+	-	+++
Gram negative	*P. vulgaris*	++	++	-	+++
	E. coli	+++	++	-	+++
	P. aeruginosa	++	++	-	+++
	S. typhi	++	++	-	+++

ZOD: - no inhibition, + zone of diameter less than 1mm, ++ zone of diameter between 2 to 5 mm, +++ zone of diameter between 5 to 10 mm, ± indicate standard error of means, n=3.

B) Antibiotic susceptibility studies of Lp, Lr and La

Lp as the intact cells, compared to the supernatant and lysate shows resistance to Penicillin G (10 µg), Ampicillin (10 µg), Streptomycin (10 µg), Tetracycline (30 µg), Chloramphenicol (30 µg), Nalidixic acid (30 µg), Azithromycin (15 µg), Gentamicin (10 µg), Ciprofloxacin (10 µg) and Vancomycin (30 µg). While it is sensitive to Kanamycin (30 µg), and Erythromycin (10 µg) (Table 6.4.1). Similarly isolate of Lr as intact cells, compared to the supernatant and lysate shows resistance to Ampicillin (10 µg), Streptomycin (10 µg), Chloramphenicol (30 µg), Nalidixic acid (30 µg), Azithromycin (15 µg), Ciprofloxacin (10 µg) and Vancomycin (30 µg)

(Table 6.4.2). The reference strain of La is found mostly sensitive to all antibiotics in cases of the intact cells, supernatant and lysate form. (Table 6.4.3)

Table. 6.4.1. Antibiotic susceptibility of *L. plantarum*

Antibiotics	Concentration (µg/disc)	Lp		
		Whole cell	Supernatant	Lysate
Tetracycline	30	R	R	R
Streptomycin	10	R	R	R
Gentamicin	10	R	R	R
Ampicillin	10	R	R	R
Nalidixic acid	30	R	R	R
Kanamycin	30	MS	S	MS
Erythromycin	10	S	S	S
Ciprofloxacin	10	R	R	R
Penicillin G	10	R	R	R
Vancomycin	30	R	R	R

R- resistant, MS- moderately sensitive, S- sensitive

Table. 6.4.2. Antibiotic susceptibility of *L. rhamnosus*

Antibiotics	Concentration (µg/disc)	Lr		
		Whole cell	Supernatant	Lysate
Tetracycline	30	MS	S	MS
Streptomycin	10	R	R	R
Gentamicin	10	S	S	S
Ampicillin	10	R	R	R
Nalidixic acid	30	R	R	R
Kanamycin	30	MS	S	MS
Erythromycin	10	S	S	S
Ciprofloxacin	10	R	R	R
Penicillin G	10	MS	S	MS
Vancomycin	30	R	R	R

R- resistant, MS- moderately sensitive, S- sensitive

Table. 6.4.3. Antibiotic susceptibility of *L. acidophilus*

Antibiotics	Concentration (µg/disc)	La		
		Whole cell	Supernatant	Lysate
Tetracycline	30	S	S	S
Streptomycin	10	MS	S	MS
Gentamicin	10	MS	S	S
Ampicillin	10	MS	S	MS
Nalidixic acid	30	S	S	S
Kanamycin	30	MS	MS	MS
Erythromycin	10	S	S	S
Ciprofloxacin	10	S	S	S
Penicillin G	10	S	S	S
Vancomycin	30	S	S	S

R- resistant, MS- moderately sensitive, S- sensitive

C) Auto-aggregation studies of Lp, Lr and La

The auto-aggregation phenomenon was observed by phenotypic methods and was demonstrated by equation 6.5. The lyophilised Lp (F19) formulation shows 96 ± 1.4% of auto-aggregation, which is observed nearly the same in cases of all (F19 - F24) Lp formulations. While, the lyophilised Lr (F13) formulation shows 90 ± 1.5% of auto-aggregation, which is observed nearly the same in cases of all (F14 - F18) Lr formulations (Table. 6.5). Similarly, the lyophilised Lp (F1) formulations show 96 ± 1.5% of auto-aggregation, which is observed nearly same in cases of all

(F2 - F4) Lp formulations. While, the lyophilised Lr (F5) formulation after spray dry shows 90 ± 1.4% of auto-aggregation, which is observed nearly same in cases of all (F6 - F8) Lr formulations (Table. 6.5). While, the lyophilised La (F9) formulation after spray dry shows 16 ± 2.4% of auto-aggregation, which is observed nearly the same in cases of all (F10 - F12) La formulation. While, the lyophilised La (F25) formulation after lyophilisation shows 16 ± 1.1% of auto-aggregation, which is nearly same in cases of (F26 - F30) all La formulation (Table. 6.5). The similar type of work was observed in case of *Clostridium butyrium* broth showing precipitate even after the wash with PBS solution [22]. The auto-aggregation helps the LAB to prevent the adhesion of pathogenic microbes to gastrointestinal mucus. It concludes that spray dry and lyophilisation techniques protect LAB in the form of formulations and maintain its auto-aggregation properties.

Table no. 6.5. Auto-aggregation properties of Lactobacillus spray-dried formulations

Bacterial isolate	Formulations	% Auto-Aggregation
Lp	F1	96 ± 1.5
	F2	96 ± 1.7
	F3	96 ± 2.4
	F4	96 ± 1.4
Lr	F5	90 ± 1.4
	F6	91 ± 2.4
	F7	90 ± 1.8
	F8	90 ± 2.2

La	F9	16 ± 2.4
	F10	16 ± 1.1
	F11	16 ± 2.2
	F12	16 ± 1.7

± indicate standard error of means, n=3.

Table no. 6.6. Auto-aggregation properties of Lactobacillus lyophilized formulations

Bacterial isolates	Formulations	% Auto- aggregation
Lr	F13	90 ± 1.5
	F14	90 ± 1.7
	F15	90 ± 2.4
	F16	90 ± 1.4
	F17	91 ± 2.1
	F18	90 ± 1.1
Lp	F19	96 ± 1.4
	F20	96 ± 2.4
	F21	96 ± 1.8
	F22	96 ± 2.2
	F23	96 ± 1.7
	F24	96 ± 0.8

La	F25	16 ± 1.1
	F26	16 ± 2.2
	F27	16 ± 1.7
	F28	16 ± 2.4
	F29	16 ± 1.5
	F30	16 ± 1.3

± indicate standard error of means, n=3.

D) Surface hydrophobicity analysis of Lp, Lr and La

Hydrophobicity study was conducted to determine the relationship between mucosal adhesion by the LAB and its physiochemical properties. The cell surface hydrophobicity phenomenon is observed by the phenotypic method and demonstrated by equation 6.6. Lp (F19) after lyophilization shows 6.1 ± 1.26% of cell surface hydrophobicity, which is nearly the same in the cases of F19 - F24 formulations. While Lr (F13) after lyophilization shows 7.2 ± 1.51% of cell surface hydrophobicity, which is nearly the same in the cases of F14 - F18 formulations (Table. 6.7). Similarly, Lp (F1) after spray dry encapsulation shows 7.1 ± 1.21% of cell surface hydrophobicity, which is nearly the same in the cases of F2 - F4 formulations. While Lr (F5) after spray dry encapsulation shows 6.0 ± 1.42% of cell surface hydrophobicity, which is nearly the same in the cases of F6 - F8 formulations (Table. 6.5). While, La (F9) after spray dry encapsulation shows 2.0 ± 1.18% of cell surface hydrophobicity, which is nearly the same in the cases of F10 - F12 formulations. While La (F25) after lyophilization shows 1.9 ± 1.40% of cell surface hydrophobicity, which is nearly the same in the cases of F26 - F30 formulations (Table. 6.8).

Ossowski et al. tried to find such a relationship between adhesion and hydrophobicity, none of them were successful [23]. The same results were observed in a case of both LAB, thus not showing any relationship between hydrophobicity and adhesion abilities.

Table 6.7. Percentage of hydrophobicity of LAB from spray-dried formulations

Bacterial isolate	Formulations	% Cell-surface hydrophobicity
Lp	F1	7.1 ± 1.21
	F2	7.0 ± 0.16
	F3	6.9 ± 1.11
	F4	7.2 ± 1.24
Lr	F5	6.0 ± 1.42
	F6	6.1 ± 1.12
	F7	6.0 ± 1.42
	F8	6.0 ± 1.52
La	F9	2.0 ± 1.18
	F10	2.1 ± 1.15
	F11	2.0 ± 1.24
	F12	2.0 ± 1.12

± indicate standard error of means, n=3.

Table 6.8. Percentage of hydrophobicity of LAB from the lyophilised formulations

Bacterial isolates	Formulations	% Cell-surface Hydrophobicity
Lr	F13	7.2 ± 1.51
	F14	7.0 ± 0.56
	F15	6.9 ± 1.21
	F16	7.1 ± 1.54
	F17	7.2 ± 1.15
	F18	6.9 ± 1.42
Lp	F19	6.1 ± 1.26
	F20	6.0 ± 1.05
	F21	6.1 ± 1.12
	F22	6.0 ± 1.16
	F23	6.0 ± 1.05
	F24	6.0 ± 1.52
La	F25	1.9 ± 1.40
	F26	2.1 ± 1.15
	F27	2.0 ± 1.14
	F28	2.0 ± 1.24
	F29	2.1 ± 1.21

	F30	1.9 ± 1.40

± indicate standard error of means, n=3.

***E) In-vitro* red snapper fish assay of Lp, Lr and La**

Adherence abilities of LAB strains of different formulations of spray dry (F1 - F12) and lyophilization (F13 - F30) granules i.e. Lp and Lr were tested in the red snapper fish intestinal mucus. The result shows that Lp adhered ($p<0.05$) significantly higher than Lr and La. A significant difference is observed in the case of adherence properties of Lp and Lr after spray dry encapsulation (Fig. 6.7) and lyophilisation due to the use of different lyoprotective excipients as seen in fig. 6.8. Similarly, various adhesion proteins, responsible for cell auto-aggregation and adhesion was previously studied in LAB [10]. Thus similar kind of adhesion process is demonstrated in Lp and Lr. The adherence properties of Lp after spray dry shows significantly ($OD_{640} = 0.61 \pm 0.83$) greater adhesion as compared to Lr ($OD_{640} = 0.32 \pm 0.93$), while La which is used as reference standard shows negligible cell adhesion ($OD_{640} = 0.15 \pm 0.53$).

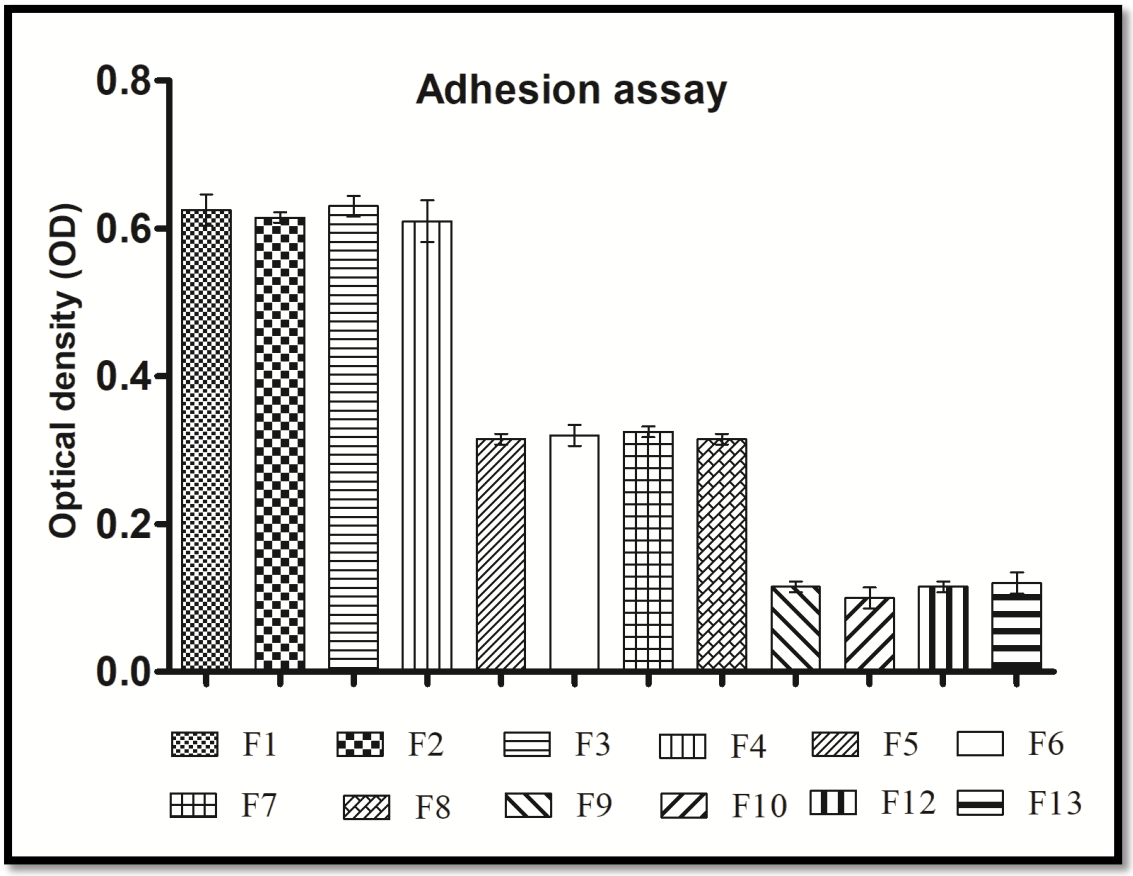


Figure 6.7. Cell adhesion of Lactobacillus formulated by spray dry method

± indicates standard error of means, n=3.

Similarly, the adherence properties of Lp after lyophilisation shows significant (OD_{640} = 0.61 ± 1.53) greater adhesion as compared to Lr (OD_{640} = 0.35 ± 0.73), while La which is used as reference standard shows negligible cell adhesion (OD_{640} = 0.15 ± 0.78)

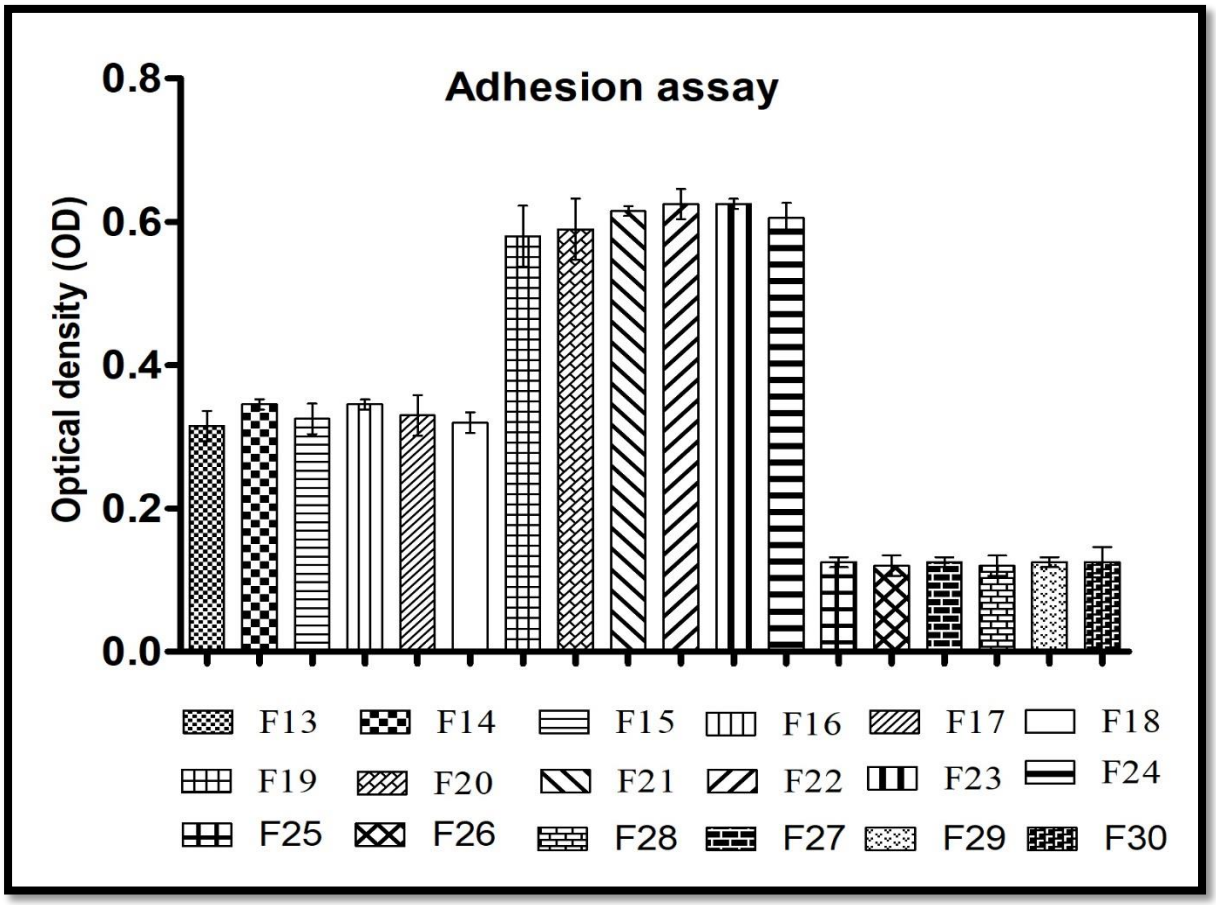


Figure 6.8. Cell adhesion of Lactobacillus formulated by spray dry method, ± indicates standard error of means, n=3.

Georgia Saxami et al., studied the adhesion properties of *L. pentosus* and *L. plantarum*, isolated from table olives [12]. They demonstrated that both strains exhibited higher adhesion rates to Caco 2 colon cancer cells and compared that to control group of *L. casei*.

6.4. Conclusions

The present chapter focuses on the antioxidant abilities of the isolated Lactobacillus. *L. plantarum* as lysate shows the strongest antioxidant activity as compared to the rest other Lactobacillus strains. Further, *L. plantarum* as intact cell shows optimal antibiotic susceptibility activity i.e. resistance against most of the available antibiotics. *L. plantarum* shows positive antagonistic activity against both Gram-positive and Gram-negative microorganisms, mostly as the intact cells as compared to lysate and supernatant form. This strain of *L. plantarum* shows

strongest auto-aggregation and hydrophobicity activity; contributing to the antioxidant mechanism as compared to the other two LAB.

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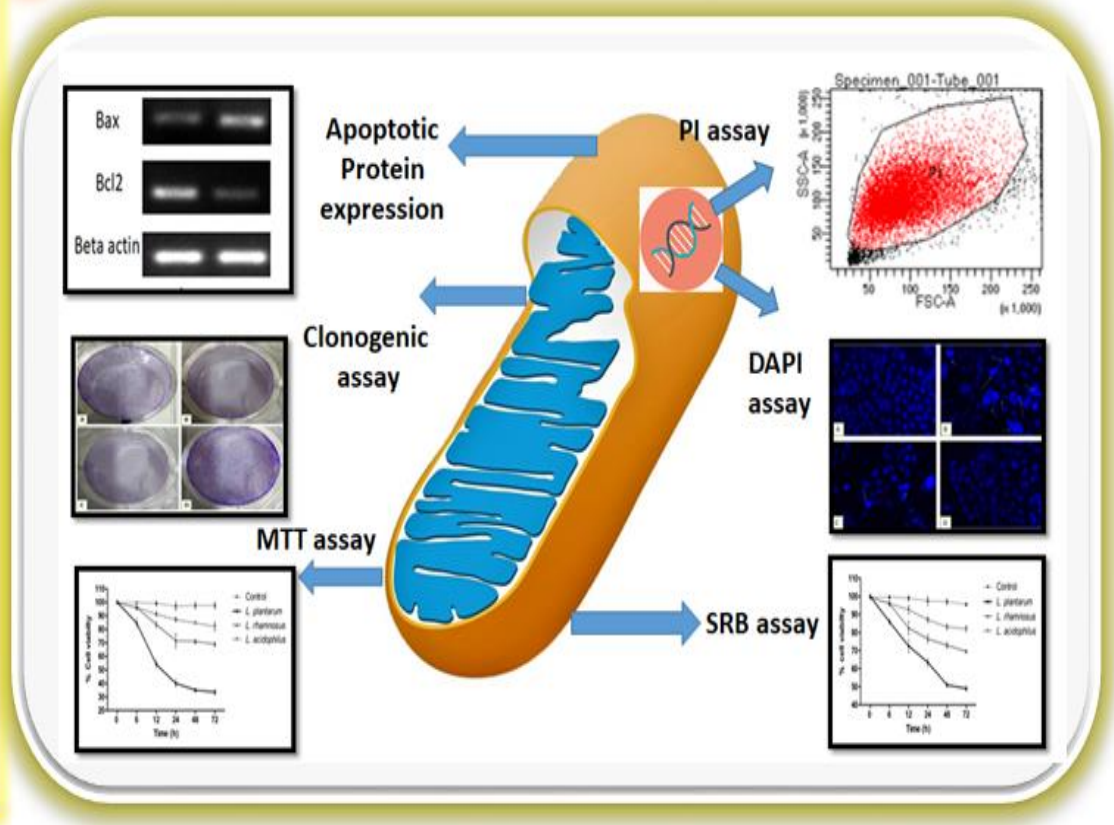
Chapter 7

Many medicines few cures- Benjamin Franklin

In-vitro colon cancer cell line cytotoxicity and anti-colon cancer activity of Lp, Lr, and La



7.1 Introduction

The pharmacology research, for the evaluation of the toxicity, is become vital in regards to the neutraceuticals products development [1]. The cytotoxicity studies of the nutraceutical products, especially the probiotics formulation are gaining importance due to its industrial application. The interaction of the probiotics cells with the living host cells may rarely precipitate toxicity. The interaction of the Lactobacillus in pathophysiological conditions such as cancer is gaining importance as an alternative source of functional food [2].

The prime requisite in using Lactobacillus as functional food is the cancer healing ability of it by the precipitating the inflammatory responses in cancerous cells without affecting the host immunological system [3]. To understand the interactions between host cells, cancerous cells, and probiotics; *in-vitro* studies by using various toxicity assay are recommended [4]. Very few research papers are published in that regards since 2011- 2019 including the cytotoxicity assays involving the nuclear fragmentation to apoptosis studies. The use of the cell lines i.e. primary cell culture act as a mimic model to understand the pharmacological role of pharmaceutical formulations. The dose and time-dependent cell viability studies such as MTT and SRB assays on cancer cell lines give a brief idea about the toxicity, dose regimen, and effectiveness of any pharmaceuticals and nutraceuticals products [5]. This ideation gives the direction whether to carry out the *in-vivo* animal model studies or not [6].

The genetic analysis involving the up-regulation and down-regulation of the proteins by a specific gene plays a vital role in understanding the central dogma of the pathophysiological condition of cancerous host cells. The study involving the interaction of the cancerous cell with Lactobacillus are indicated by means of gene-protein regulation studies [7]. The level of apoptotic and anti-apoptotic proteins regulated by the gene Bcl2 and Bax helps in determining the death of the cancerous cells and acts as a fingerprint technique in the field of cancer research. The cell death

observed in the case of a cancerous cell line by the Lactobacillus is mostly undefined. The cancer cell death hypothesis proposed by the researchers includes, the apoptosis and/ or by necrosis pathways [8]. Similarly, the clonogenic assay along with annexure V/PI and DAPI are proven important to identify the exact reason behind the type of cancerous cell death [9,10]. On this background, the following chapter deals with the cytotoxicity studies of Lactobacillus on the HCT 115 colon cancerous cell line. *L. acidophilus* which was obtained from the NCIM, Pune is used as the reference control group.

7.2 Experimental

7.2.1 HCT 115 cell culture and cell-free lyophilized filtrate preparation

The human colon cancer cell line HCT 115 are developed as monolayers by maintaining it in RPMI (Roswell park memorial institution medium). The procurement of these cells was carried out from NCCS Pune. These media are augmented with 100 U/ml streptomycin-penicillin and 11% temperature maintained heated killed fetal bovine serum (FBS). The cells were maintained in a humidified atmospheric condition of 4.6% CO_2 at around 36 ºC (approximately). After 80–85% confluence, cells were treated with the cell-free lyophilized filtrate of the bacterial samples [11,12].

The Lp, La and Lr cultures of Lactobacillus were grown in MRS broth at 37 ºC for overnight with a concentration of 1 X $10^{7\sim8}$ cfu count/ml. Later, these cultures was centrifuged at 5100 x g for 5.5 min at 4 ºC to get pellets. The formed cell pellets were washed with PBS prior to ultrasonic disruption in a cooling ice bath (4 ºC) for 15 min at an interval of 28 s. The formed supernatant is passed through the 0.22 µm pore microfilter (Himedia, USA). The sterilized filtrate of supernatant was freeze-dried and various concentration was used for cell viability assays [13].

7.2.2 Investigation of cytotoxicity of Lactobacillus

A. Sulforhodamine B (SRB) assay of Lp, Lr, and La

The cellular protein content is measured in SRB assay by determination of cell density [14]. SRB assay was carried out according to the protocol defined by Tiptiri *et al*., with slight modifications [14]. In brief, the cell in the concentration of $1\times 10^{5\text{-}6}$ cells/ml was incubated for 24 h using 96- well microtitre plate. After incubation, the cells were treated with the cell-free lyophilized filtrate of the Lp, Lr and La in a concentration of 10, 20, 30, 40 and 50 (µg/ml). Then, the total medium was incubated at 38 ºC in a 5% CO_2 atmospheric conditions for 24 h, 48 h, and 72 h. After the incubation period, the formed cell monolayers were fixed with 9.9% (w/v) trichloroacetic acid. Further, it was stained for 30 min with SRB and for removing the excess dye, 1% (v/v) acetic acid was used. The OD was calculated at 500 nm by dissolving the protein-bound dye in 9.9 mM Tris base solution using a microplate reader for determination of the cell viability. The % cell viability was calculated by comparing with the values of the control well without any LAB samples and using equation 7.1. La sample was used as a reference control group.

% Cell viability = Absorbance of the cell under treatment/ Control cell absorbance (7.1)

B. MTT assay of Lp, Lr, and La

The anchorage-dependent viability in the form of the cell growth was determined by the colorimetric assay using [3-(4-5-dimethylthiazol-2yl-)-2, 5-diphenyl tetrazolium bromide] MTT reagent [13]. In MTT assay, the purple formazan color of the reduced MTT dye is found directly proportional to viable cell number quantitated by the spectrophotometric study. In brief, 9,000–10,500 cells of HCT 115 were seeded (triplicates) in the 96-well flat-bottomed tissue-culture plates for 24 h. After 24 h, the cells were treated with the cell-free lyophilized filtrate of the bacterial samples of Lp, La and Lr in a concentration of 10, 20, 30, 40 and 50 (µg/ml). The negative control group kept for the assay consist of non-inoculated RPMI

medium. The cells washed with the PBS solution after 24 h post- treatment were inoculated with 5 mg/ml solution of the MTT in PBS. Further, the cells were incubated for 4 h at 37 ºC for the mitochondrial dehydrogenase activity which was later visualized by the purple formazan crystals formation. Finally, in each well 120 µl of DMSO (dimethyl sulfoxide) was added and incubated for 28 min at 37 ºC. By the spectrophotometric method using a microplate reader, the color intensity was measured at 570 nm. All the experiments executed in triplicates (n=3), and the data were represented as percent viability compared to control**.**

7.2.3 Nuclear staining with DAPI of Lp, Lr, and La

The cell-specific apoptotic process is characterized by DAPI assay. In HCT 115 cells, the analysis of nuclear fragmentation along with the chromatin condensation was done by DAPI staining using fluorescence microscopy [10,15]. After treatment of 70–80% confluent HCT 115 cells with cell-free lyophilised filtrate of Lp, Lr and La (50 µg/ml in RPMI) for 24 h the cells were quickly washed with ice-cold PBS and fixed in ice-chilled acetone: methanol (1:1) mixture for 10 min at 4 ºC in the dark. The cells after washing with ice-cold PBS were incubated for 20 min with the DNA-specific fluorochrome, DAPI. The ice-cold PBS wash was carried out to remove the excess of DAPI. Finally, the cells were observed using a fluorescence microscope at 20-40 X resolution. La sample was used as a reference control group

7.2.4 Clonogenic assay of Lp, Lr, and La

The proliferation and survival studies of the cells are performed by the clonogenic assay [7]. For this, HCT 115 cells were incubated for 24 h by seeding in 35 mm plates and then treated with 50 µg free lypholized filtrate of Lp, Lr and La for next 24 h. La sample was used as reference control group. Thereafter, the medium was kept incubators for 5-6 times doubling by replacing with fresh medium. The medium was removed after colony formations and was air dried. Further, it was stained with 0.18% solution of crystal violet. Then, the wells were treated with the distilled water, and colonies were counted by a gel documentation system.

Experiments were carried out in triplicates, and the data are represented as a number of colonies. PBS treated group was kept as a control for the study.

7.2.5 Annexin-V & propidium iodide (PI) assay of Lp, Lr, and La

HCT 115 cells were cultured in a dish of 60 mm and further, treated with the cell-free filtrate of Lp, Lr and La (500 µl). After a 24 h incubation period, the harvested cells were washed with cold PBS. 1% Annexin binding buffer solution was prepared. 5 µl of the 1 mg/ml PI stock solution was used to prepare a diluted 100 µg/ml working solution of PI. The final solution along with the inoculated cell was centrifuged and the cells were suspended in 1X Annexin-binding buffer after discarding the supernatant solution. The final volume of 100 µl of cells per assay of buffer was prepared by diluting the 1X Annexin-binding buffer to ~1 × $10^{5\sim6}$ cells/ml. 1 µl 100 µg/ml PI working solution and 6 µl of Alexa Fluor® 488 Annexin V were added to form 100 µl of cell suspension. Later, the cells were kept at room temperature for about 12 min for the incubation. Finally, the cells were inoculated with the 390~410 µl of 1X Annexin-binding buffer and were placed in an ice bath. The stained cells were evaluated by measuring the fluorescence emission at 520 nm using the flow cytometry [10].

7.2.6 Semi-quantitative PCR of Lp and Lr

A) Isolation of total cellular RNA

From the cell lysate, the total cellular RNA was isolated from treated and untreated HCT 115 cells with Trizol isolation method [7]. Briefly, the media was discarded from the HCT 115 cells cultured in a plate after the incubation from the incubator after 24 h. As per the manufacturer instruction, the Trizol reagent was added to the culture plates (595~605 µl per 60 mm dish). The homogenous solutions were developed from the lysate solution using an aseptic pipette. The nucleoprotein complex was dissolved by taking the mixture in 1.5 ml micro-centrifuge tube by incubating the samples for 5 min at 34 ^{o}C. Then, the combination of 200 µl of chloroform in 1 ml of Trizol reagent was inoculated into the mixture by shaking for

a period of 25 s, further kept for 10 min at 34 ^{o}C. The aqueous phase of the mixture was removed by centrifugation of the final solution at 12000 rpm for 18 min at 4 ^{o}C. The RNA raw product was obtained by adding 500 µl of isopropanol to the aqueous solution in the form of a precipitate. The RNA pellets were obtained by further centrifugation of the final mixture at 12000 rpm for 12 min at 4 ^{o}C. The generated RNA pellets were again washed with 1ml of 76% ethanol followed by 10500 rpm centrifugation at 4 ^{o}C for 5min. The obtained RNA pellets were dissolved in 20 µl of nuclease-free water and stored at -40 ^{o}C for future processing [10].

Fig 7.1. Bio-Rad T-100 PCR

B) cDNA preparation

20 µl reaction mixture was prepared by using 2 µg of total RNA (drug-treated and control), 2 µl of oligo (dT) along with the 13.7 µl addition of nuclease-free water. The formed sample mixtures were treated with the cyclic mixed conditions by incubating it at 60 ^{o}C temperature in the dry bath followed by ice water treatment for 600 s. Further, the ice treated mixture was kept at 42 ^{o}C temperature for 10 min and further processed in dry ice conditions with 4µl of 5X reverse transcriptase buffer, 0.2 µl of reverse transcriptase enzyme and 9.9 mM dNTP to a final volume mixture of 20 µl. The final mixtures were centrifuged at 42 ^{o}C for 1 h on a dry bath. Again the final mixtures were incubated at 95 ^{o}C for 150 s for the formation of the cDNA. The formed cDNA was stored at -40 ^{o}C for further processing [10].

Table 7.1. Reverse transcription reagents used for the cDNA synthesis

Reagents used	Volume required (µl)
Oligo dT	2
Nuclease-free water	As per requirement
5X RT buffer	4 ~ 5
Reverse transcriptase enzyme	0.2 ~ 0.3
10m M dNTP	2

C) Polymerase chain reaction

The specific genes of the cDNA were amplified by setting up a PCR reaction using the primers [14]. 25 µl gene-reaction mixtures were processed by using Bio-Rad T-100 PCR, USA.

Table 7.2. PCR: Preparation of PCR mixture

Reagents used	The volume taken (µl)
Nuclease-free water	12.75
10X Taq buffer	5
10mM dNTP	1
Forward primer	1
Reverse primer	1
Taq polymerase	0.25
cDNA synthesized	4

In thermocycler, PCR tubes were placed inside and the cycles were set as below:

1. Denaturation - 94 ^{o}C – 45 s
2. Annealing - T_m(specific to the primer used) – 45 s
3. Extension - 72 ^{o}C – 60 s
4. Final extension - 72 ^{o}C – 10 min
5. Product storage - 4 ^{o}C – infinite time

D) Band analysis

The PCR products were mixed with gel loading dye. This dye was run using 1% low melting agarose. The bands were visualized by gel documentation system [16]. The bands were normalized with the endogenous control (beta-actin) and quantified using the image-lab 5.2.1 software (Bio: rad, USA)

7.2.7 SEM analysis of Lp, Lr, and La on HCT 115 cell lines

Adhesion of Lp, Lr and La was performed as per the method described by Kumar et al. [17]. Initially, $10^{5\sim6}$ cells of HCT 115 cells per milliliter were developed in 24 well tissue culture plates. The modified Eagle's minimal essential medium (DMEM) media was augmented with 100 U/ml streptomycin-penicillin and 11% FBS [17]. The cells were maintained in a humidified atmosphere condition of 4.6% CO_2 at around 36 ºC (approximately). After 80–85% confluence adhesion assay was carried out after 3 days of post confluency. The isolates cultures of LAB were inoculated in the concentration range of about 10^6 to 10^8 cfu/ml in 1 ml DMEM medium (without antibiotics and serum). The plates were maintained in the presence of 5~6% CO_2 / 90% air atmospheric conditions. The cells were detached by using PBS from the formed monolayer by trypsinization process in which one milliliter of 0.1% trypsin solution was used and incubated for 10 min. To observe the adhesion, SEM analysis was carried out. HCT 115 cells with bacterial cells were fixed using 2.4% of glutaraldehyde in 0.1 M PBS for 1 h with at 36 ºC. The cells were rinsed thrice with PBS and maintained for 25 min with 2% osmium tetroxide in isotonic saline solution. The fixed cells were graded in a series by (30, 50, 70, 80, 90, and 100%) with ethanol after washing with PBS solution. Later, 100% hexamethyl-disilazane treatment was given to the cells and treated for 9 min with coated gold. The prepared slide was observed by the scanning electron microscope at common facilities centre; Indian Institute of Technology, Roorkee, India.

7.3 Results and Discussion

7.3.1 Choice of cell type

Each and every organ inside the human body shows different specificity and affinity with the drug. One drug having an affinity with one organ may not have the same with the others. Similarly, it is found that the toxicity produced by the drug vary from organ to organ. The major reason behind this physiological changes includes versatile environmental conditions around different cells and organ system, including the different proliferation pathway shown during the cell cycle [14].

Thus the selection of cell type or its prototype is important while studying the effects (pharmacology/toxicology) of any drug. This is studied by means of cytotoxicity assays. In the assay, the effect of the drugs also varies based upon the dosage forms and site of introduction. The effect of drugs in the form of the Lactobacillus (supernatant/lysate) on the cell line depends upon the exposure route and time. Toxicity also gets precipitated by varying the time and exposure route. Probiotics especially the Lactobacillus as formulations can be introduced into the human body through various routes. The digestive tract is the most probable entry portal for Lactobacillus formulations. A wide variety of probiotics formulation is available in the market treating various gastrointestinal disorders. In the case of neutraceutical applications, lactobacillus is often introduced into the human body through the buccal cavity route. Although they are introduced by the oral route, it important to know it's the effects on numerous cell types including serum, myocardial cell, nephrons, neurons, spleenocyte, and hepatocytes in order to study its cytotoxicity [18]. In the present investigation, we have used the HCT 115 cell lines to evaluate the cytotoxicity of the Lp and Lr by comparing it with La as a reference control group.

7.3.2 Selection of cytotoxicity assay of Lp, Lr, and La

The proper assessment of a probiotic formulation depends upon the type of the cell lines selected [19]. Cell line studies are an ethically valid, simple and reproducible form of *in-vitro* studies as compared to the animal's *in-vivo* studies.

Further, the data obtained from these *in-vitro* studies should be analytically reproducible. In cell line studies, various variables such as cells type, media conditions, alternation in temperature, varying pH, change in preparation protocol**,** and assay methodology are evaluated analytically. The toxicity profiling of the drug especially lactobacillus formulation can be studied by considering various parameters such as its genotoxicity, inflammatory responses, and oxidative stresses [20]. The results obtained from the cytotoxicity assay is very much important in analyzing the probiotics formulations.

In many of the cytotoxicity assays, the used dyes are absorbed by the cells by a various mechanism which helps in understanding the pharmacodynamics of the drug actions. The multiple tests run on the same cell line using the same drugs gives more accurate results as compared to individual test or assay. If the *in-vitro* toxicity tests against cancer cell lines show maximum threshold pharmacological effects in the concentrations used, then the samples are forwarded to do various *in-vivo* tests in different animal models, further can be validated for human use. Once the products get approved from the FDA by the pre-clinical trials then, researchers get the green signal to carry out various phases of the clinical trials. Cytotoxicity studies and its type vary, depending upon the methods and reagents used. Thus, these studies are also depend upon the types of cell lines used. Some studies deal with the cell morphology and its structure, while other deals with the DNA damage levels and its types. These studies are evaluated based upon the amount of drug used and its required incubation time [21]. Sometimes, variable results are obtained due to the interaction of the dye with the growth media instead of the drug; that is nullified by the repetition of the experimentations in different variable conditions.

Thus, all the potential interferences are determined before going for any kind of the assay in order to get the accurate results in cytotoxicity experimentations, which is practically difficult in the animal models studies. Therefore, cell lines studies are always an unswerving way to screen the cytotoxicity effects of the drug/ probiotics/ Lactobacillus comprising of two or more independent assays in order to get accurate reliable results [4]. The principle behind the different *in-vitro* toxicity assays varies from one another along with the type of methodology used [22] (Fig. 7.2).

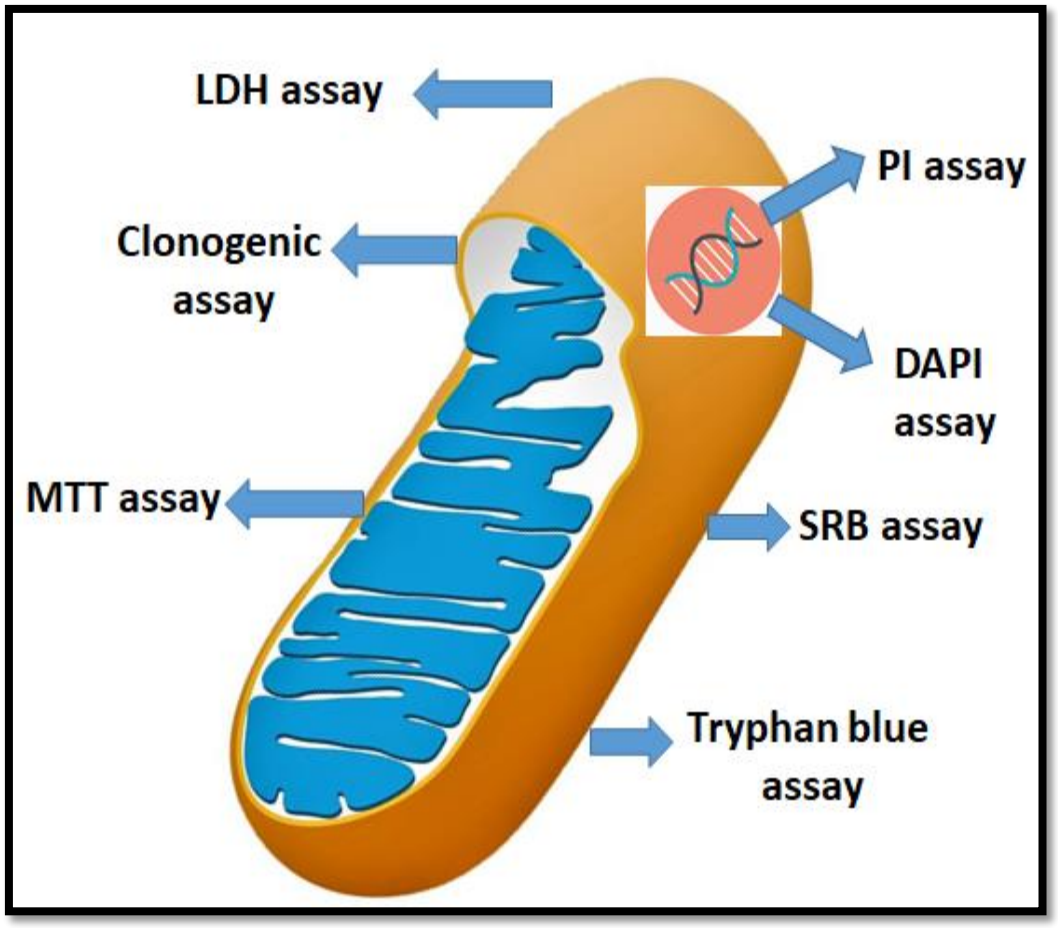


Fig 7.2. Different *in-vitro* toxicity assays

**** Sulphorhodamine B (SRB) assay -** Total protein from the cell in the form of its content is measured by SRB assay, related directly to its cell viability.

**** 3-(4,5-Dimethylthíazol-2-yı)-2,5-Diphenyltetrazolium bromide (MTT) assay –** The cell proliferation rate is measured by observing the reduction in metabolically active cells and its levels.

**** Lactate dehydrogenases assay** – The damage to the membranous layer of the cells is responded by the rapid release of this enzyme. The rate of absorption is evaluated by dying damaged cell.

**** Tryphan blue assay** – In this assay, the cell viability is determined depending upon the uptake of the dye. Only dead cells get stained excluding the living cells.

**** Bromodeoxyμridine (BrdU) assay** - It is a thymidine analog nucleoside with the synthetic origin. In this assay, the proliferation of the cell triggered by the add-on of BrdU into DNA is evaluated by the colorimetric analysis. The quantitative addition of BrdU in the cells is proportional to the proliferation rate of the cellular matter.

**** Propidium iodide (PI) assay -** PI is a dye used to distinguish the living and dead cells; acts on membrane impairing dead cells. It is related to the apoptotic pathway determination of the dead cells, not affecting the living cells.

7.3.3 Cytotoxicity studies of Lp, Lr, and La

A) SRB assay on HCT 115 cell line

The amino xanthene dye used in SRB assay is an anionic ion called as the Sulforhodamine B (SRB), which provides the direct sensible response with the protein molecule in form of the basic amino group by electrostatic complex interactions. All the living cells produce proteins, even the cancerous cells. The decrease in the total protein contents of the cancer cell lines by the introduction of Lactobacillus content can be studied to determine the viability of cancer cell lines. The death of the cells, stop the cellular activity resulting in the breakdown of cellular proteins into amino acids producing no stains.

On this background, the viability study conducted by SRB assay on HCT 115 cell line shows a decrease in percentage cell viability an approximately 64% in case of Lp for 50 μg/ml concentration with respect to control group (equation 7.1). Similarly, the cell viability results obtained in the case of Lr and La shows 74% and 88% viability of HCT 115 cell line for 50 μg/ml with respect to the control group (Fig. 7.3)

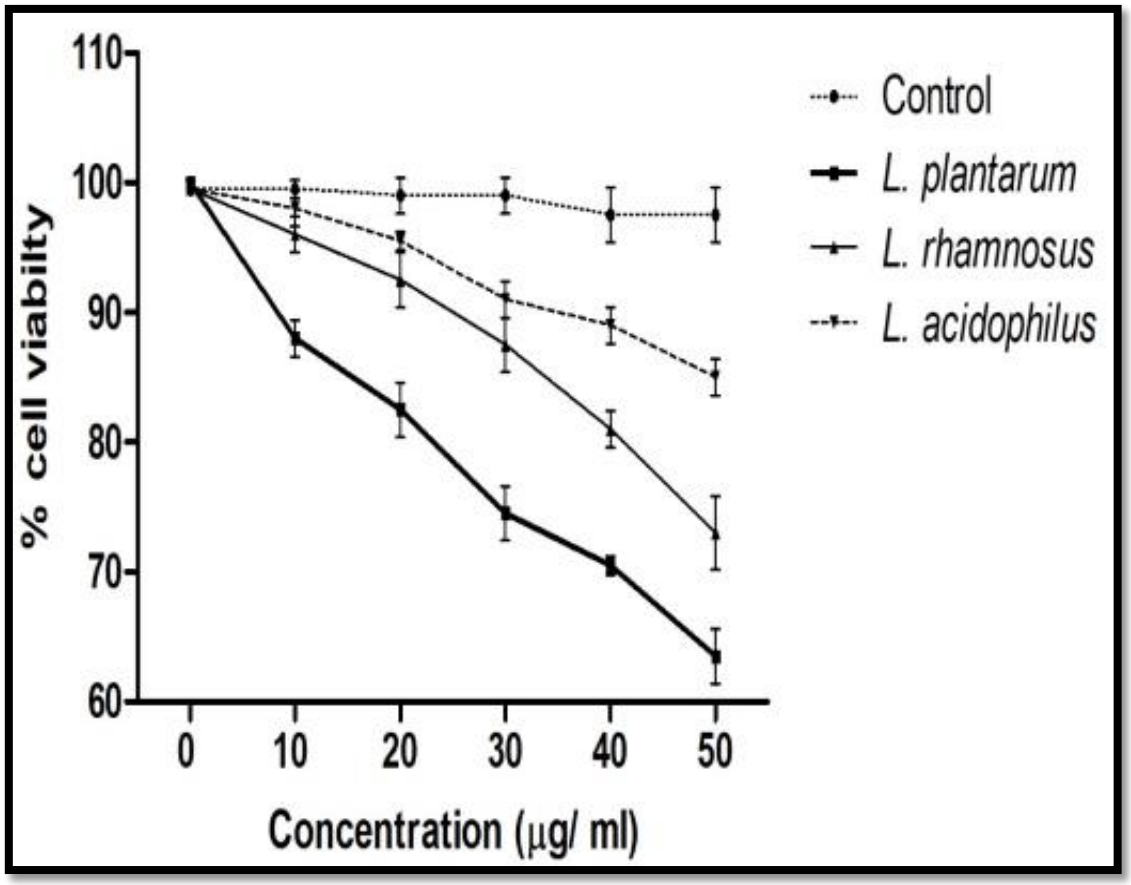


Fig.7.3. Effects of the cell-free lyophilized filtrate of LAB on the viability of HCT 115 cell line for various concentration by SRB assay. Values are expressed as viability mean ratio ± *SD**, n = 3.

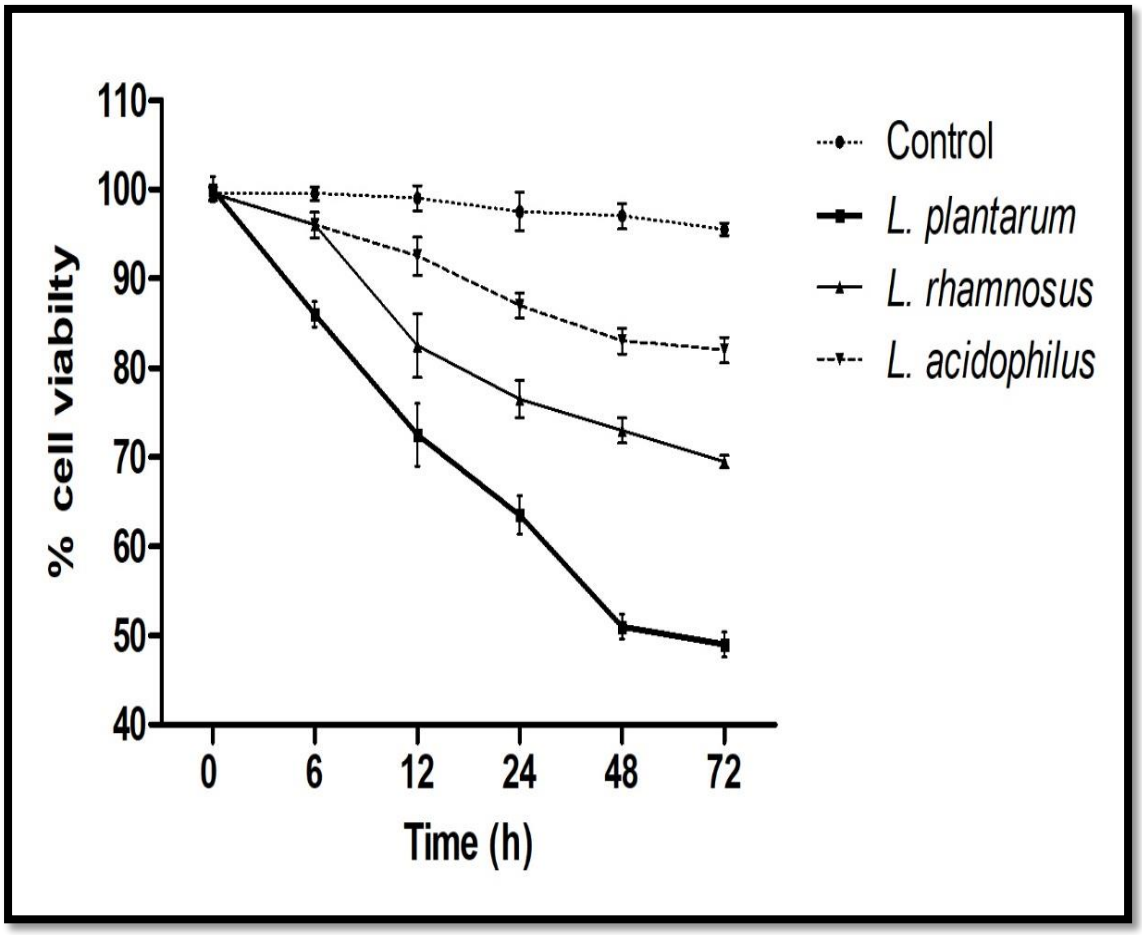


Fig.7.4. Effects of the cell-free lyophilized filtrate of LAB on the viability of HCT 115 cell line for various incubation time by SRB assay. Values are expressed as viability mean ratio ± *SD**, n = 3.

Time-dependent SRB assay was carried out for a period of 24 h, 48 h, and 72 h. The study reveals that the cell viability decreased with time. Lp shows a decrease in viability approximately from 64% at 24 h, 52% at 48 h to 48% after 72 h as compared to the control group. Similarly, the cell viability got decreases with the time in the case of Lr with an approximate decrease in viability from 76% at 24 h, 72% at 48 h and 70% at 72 h (Fig. 7.4). While, very negligible decrease in the viability of HCT 115 cell lines are observed in the case of La. Thus, both time-dependent and concentration dependent decrease in the cancer cell line viability is observed significantly in cases of Lp as compared to Lr and La by SRB assay. *L. casei* ATCC 39X was found effective against the HT 29 and CT 26 cell lines. The

cell count of 10^8 cfu/ml was found effective in reducing the cell viability of colon cancer cell lines by 52% in CT 26 cell line [14]. Thus, it shows that *L. plantarum* as an effective antiproliferative agent against HCT 115 cell line after 48 h at the concentration of 50 µg/ml.

B) MTT assay

In this assay, the MTT reagent passes through the viable cells and enters into the mitochondria, producing the dark blue/purple insoluble compound called as formazan. This is observed only in viable cells, as the yellow tetrazolium salt from MTT reagent is reduced to colored purple dye only in the living cells. These viable colored cells get homogenized and soluble because of the use of organic solvent like isopropanol resulting in the formation of the formazan. This complex colored compound is measured by the spectrophotometric method. The dead cells never show these activities thus, this assay gives the count of the living cells in the form of the viability count and determines the number of metabolically active cells.

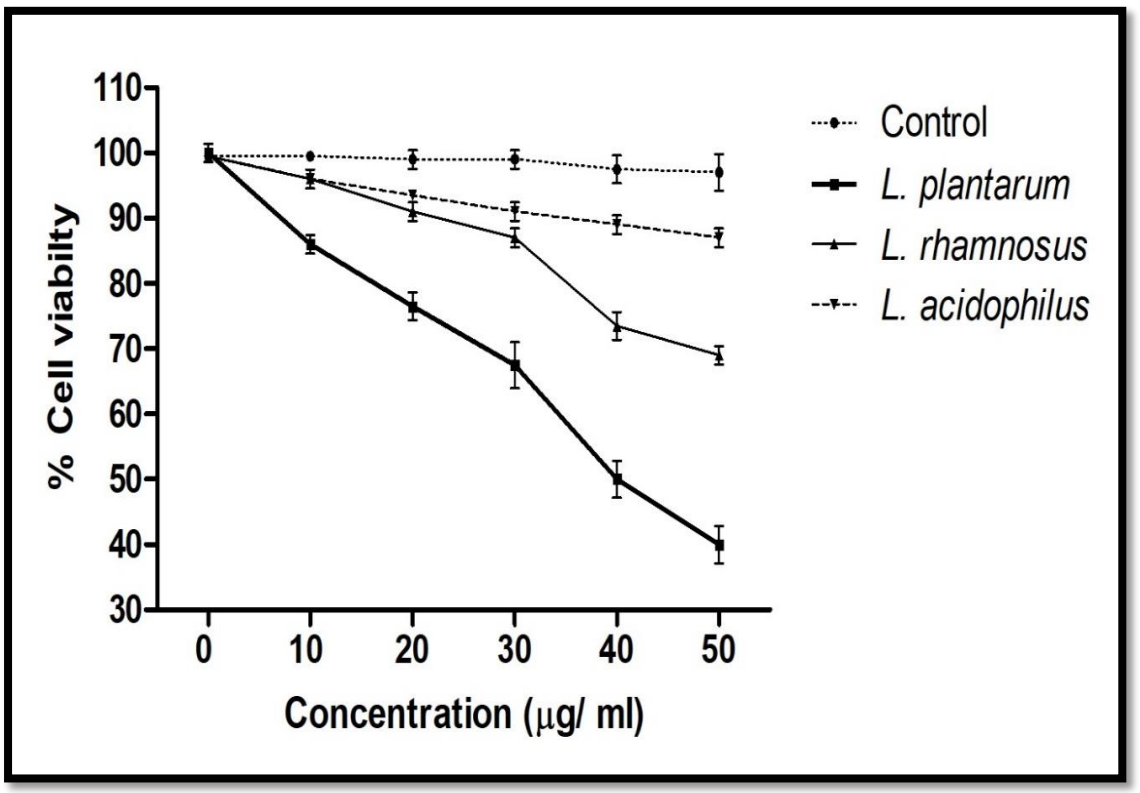


Fig.7.5. Effects of the cell-free lyophilized filtrate of LAB on the viability of HCT 115 cell line for various concentration by MTT assay. Values are expressed as viability mean ratio ± *SD**, n = 3.

The viability study conducted by MTT assay on HCT 115 cell lines shows a decrease in percentage cell viability, an approximately around 40% in case of the Lp for its highest dose of 50 µg/ml and further, showing the strongest anti-proliferative activity as compared to control group. In the case of Lp, expect the dose of 10 µg/ml the other doses from 20 µg/ml to 50 µg/ml shows the anti-proliferative activity. Similarly, the cell viability results obtained in case of Lr and La are 74% and 88% on HCT 115 cell line for its highest dose of 50 µg/ml with respect to the control group. La, which is used as the reference control group, not show any anti-proliferative activity.

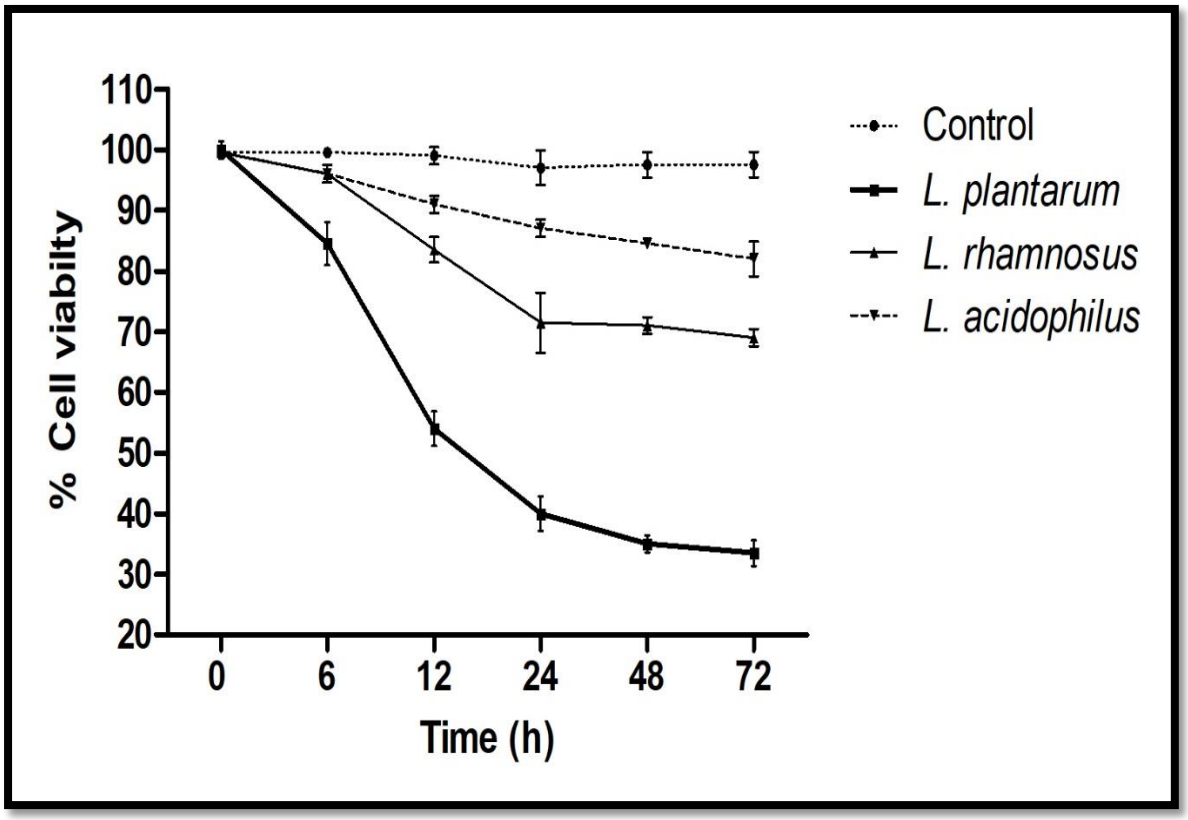


Fig.7.6. Effect of the cell-free lyophilized filtrate of LAB on the viability of HCT 115 cell line for various incubation time by MTT assay. Values are expressed as viability mean ratio ± *SD**, n = 3.

Time-dependent MTT assay is carried out for a period of 24 h, 48 h, and 72 h. The study reveals that cell viability decreases with time. Lp shows a decrease in viability count approximately from 41% at 24 h, 38% at 48 h to 34% after 72 h as compared to the control group. Similarly, the cell viabilities are decreased with the time in the case of Lr with approximately decrease in viability from 72% at 24 h, 70% at 48 h and 68% at 72 h (Fig. 7.6). While the negligible decrease in the viability in HCT 115 cell lines are observed in the case of La. Thus, both time-dependent and concentration dependent decrease in the cancer cell line viability is observed significantly in case of Lp as compared to Lr and La by MTT assay. The similar kind of the time and concentration dependant MTT studies were carried out on the *L. plantarum DGK-17* with the dose of 25 to 250 μg, showed the effective decline in the colon cancer cell line after 24 h [7]. Thus, it shows that *L. plantarum* is more effective against HCT 115 cancerous cell line at the concentration of 50 µg/ml at 48 h.

C) Mechanisms of cell death

Each and every kind of cells even cancerous cells are killed in different ways, such as apoptotic pathway, necrotic accidental pathway and alternative cell death called autophagy. The act of getting killed is called as necrosis. This death is also called as the accidental death of the cell in which the premature death of the cells may occur by the autolysis. Many times, various environmental events such as infection, toxins, traumatic reaction cause necrosis. It is an instantaneous process which occurs along with the inflammatory responses. The dead cells are destroyed by the macrophages by phagocytosis when the cells undergo the death by necrosis pathway. These cells lose the apoptotic natural signaling pathway activating various receptors resulting in the loss of the cellular integrity and scavenging by the host immune cells.

In apoptosis, series of the cascade events are activated which is slower in rate. The apoptotic process is initiated by specific immuno-chemical pathways before cell

death. It is a highly regulated process which once initiated in the cell is never reversed. It is initiated as an intrinsic pathway also called as a mitochondrial or extrinsic pathway. The stress inside the cells triggers the intrinsic pathway which is related to the release of mitochondrial proteins from the inter-membrane region. Extrinsic pathway gets activated by the binding of the ligands to the death receptors on the cell surfaces. The various phenomenal death pathways, taking place in apoptosis cells are shrinkage of cells and blebbing of the cellular membrane with apoptotic body's formation (Fig 7.7).

Autophagy is a natural recycling process of the cell called a self-eating mechanism. In this process the dysfunctional cells, organelles damaged cells and worn out cells lacking proteins are destroyed. In a diseased condition, the stress level in the cells gets elevated. These cells get isolated within double-membrane vesicle called autophagosomes which fuse with lysosomes and get killed.

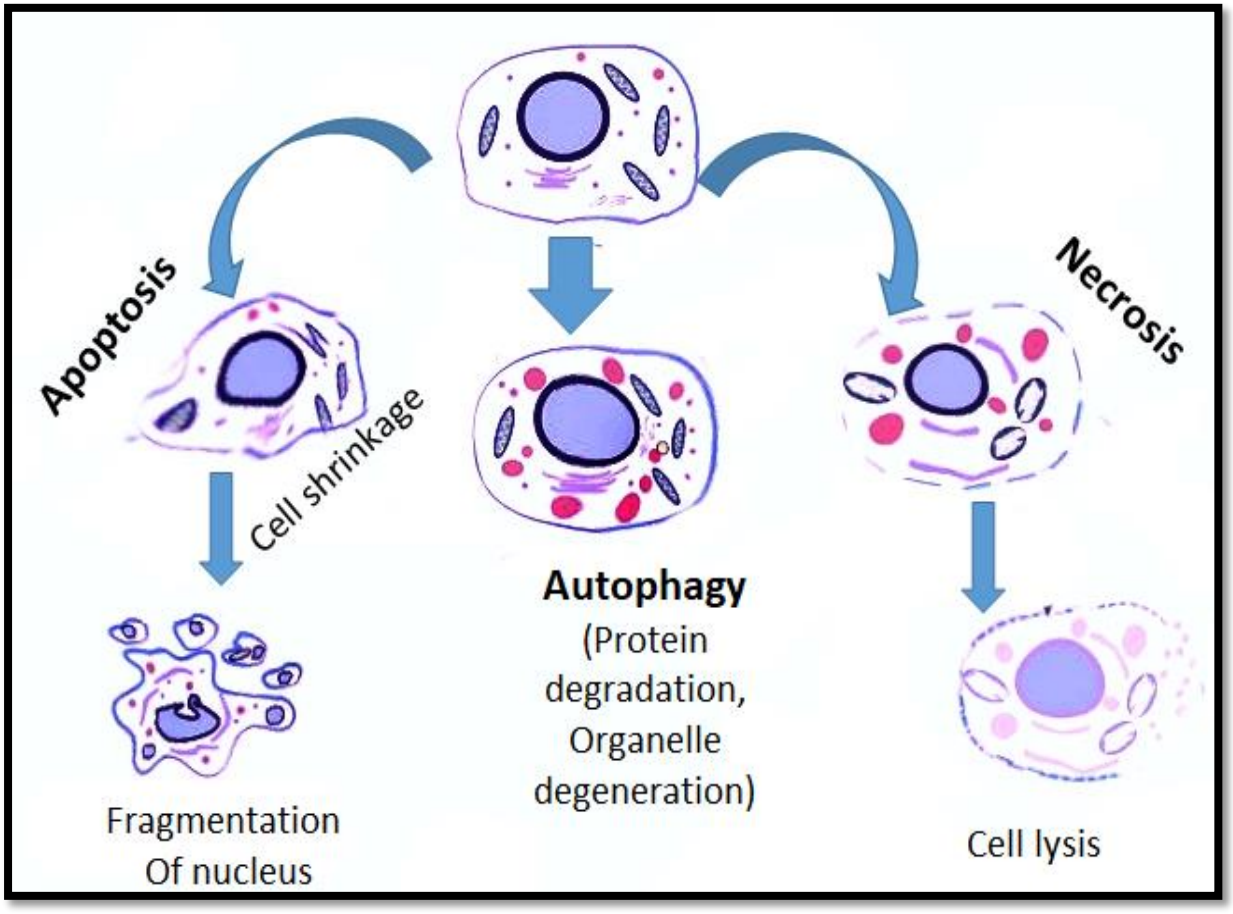


Fig 7.7. Mechanism of cell death involving apoptosis, necrosis, and autophagy.

Several different mechanisms are observed in the cancer cells induced due to the use of nutraceutical products such as probiotics formulations. The main mechanisms observed in the lactobacillus utility by the host in killing the cancer cells are a generation of excess reactive oxygen species, biomolecules disruption in the cellular membrane, alternation of the cellular hemostasis and death by the combinations of above said mechanisms.

7.3.4 DAPI analysis of Lp, Lr, and La

The cell-free lyophilized filtrate of Lp, Lr, and La was analyzed for 24 h on HCT 115 cell lines, observed by the fluorescent microscopy (Olympus, Tokyo).

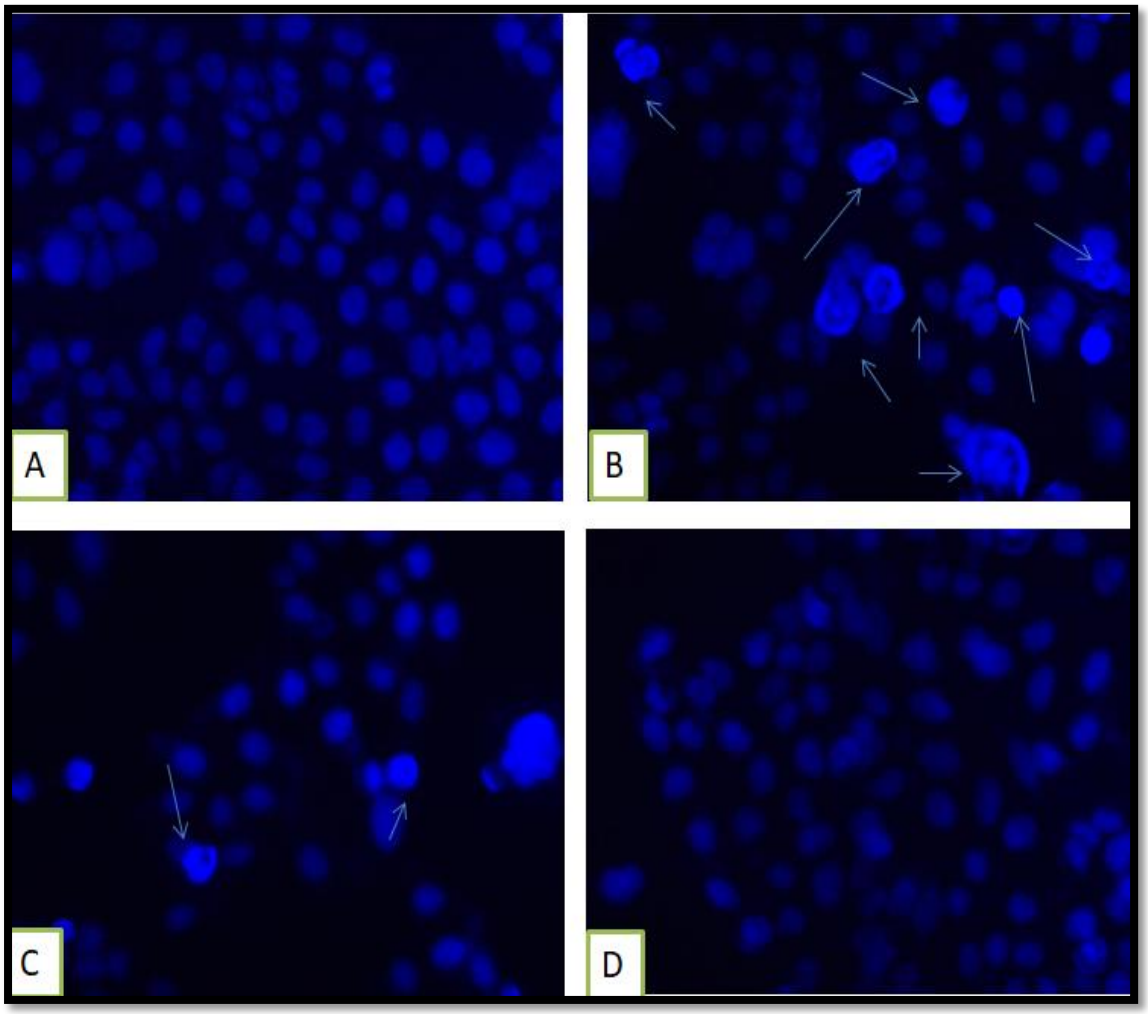


Fig. 7.8. DAPI analysis of Lactobacillus strain A) untreated cell line, B) *L. plantarum* C) *L. rhamnosus* and D) *L. acidophilus*

The significant apoptotic signals were observed in the case of Lp as compared to Lr. Non-apoptotic signals are observed in the case of the La (Fig. 7.8.D) which is used as a reference control group. The observed apoptotic cells were with fragmented condensed nuclei as compared to the intact blue color normal cells. After 24 h incubation, the Lp treated HCT 115 cells shows apoptotic shrunk cells with nuclear fragmentation pattern as compared to the Lr (Fig. 7.8.C). The other features observed in the case of the apoptotic cells are the formation of micronucleus blebbing cell membrane with the shrinkage of the nucleus in Lp (Fig. 7.8.B). While Lr shows blebbing membrane with micronucleus. Apoptosis is clearly visualized by DAPI. Previous studies conducted on *L. plantarum SBL* showed nuclear fragmentation of the Hela cell line at the concentration of 50 µg/ml with shrinkage of the nucleus [10]. On the same grounds, *L. plantarum* shows fragmentation of the nucleus with significant apoptosis cell death.

7.3.5 Clone formation assay against HCT 115 cell lines

The anticancer potential of the Lactobacillus was determined by the decrease in the clone formation. In the following study, Lp (Fig. 7.9. C) shows a significant decrease in the clone formation as compared with the Lr (Fig. 7.9. B) and La (Fig. 7.9. D).

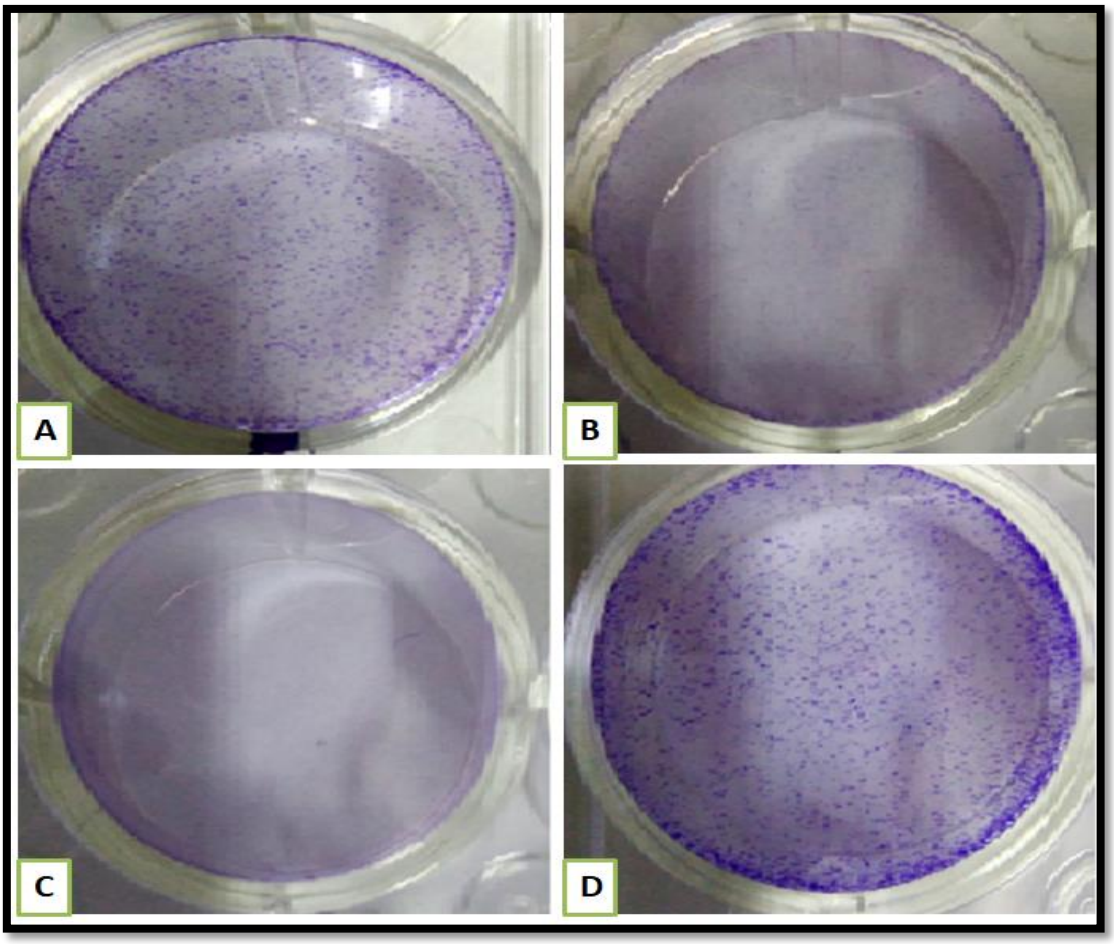


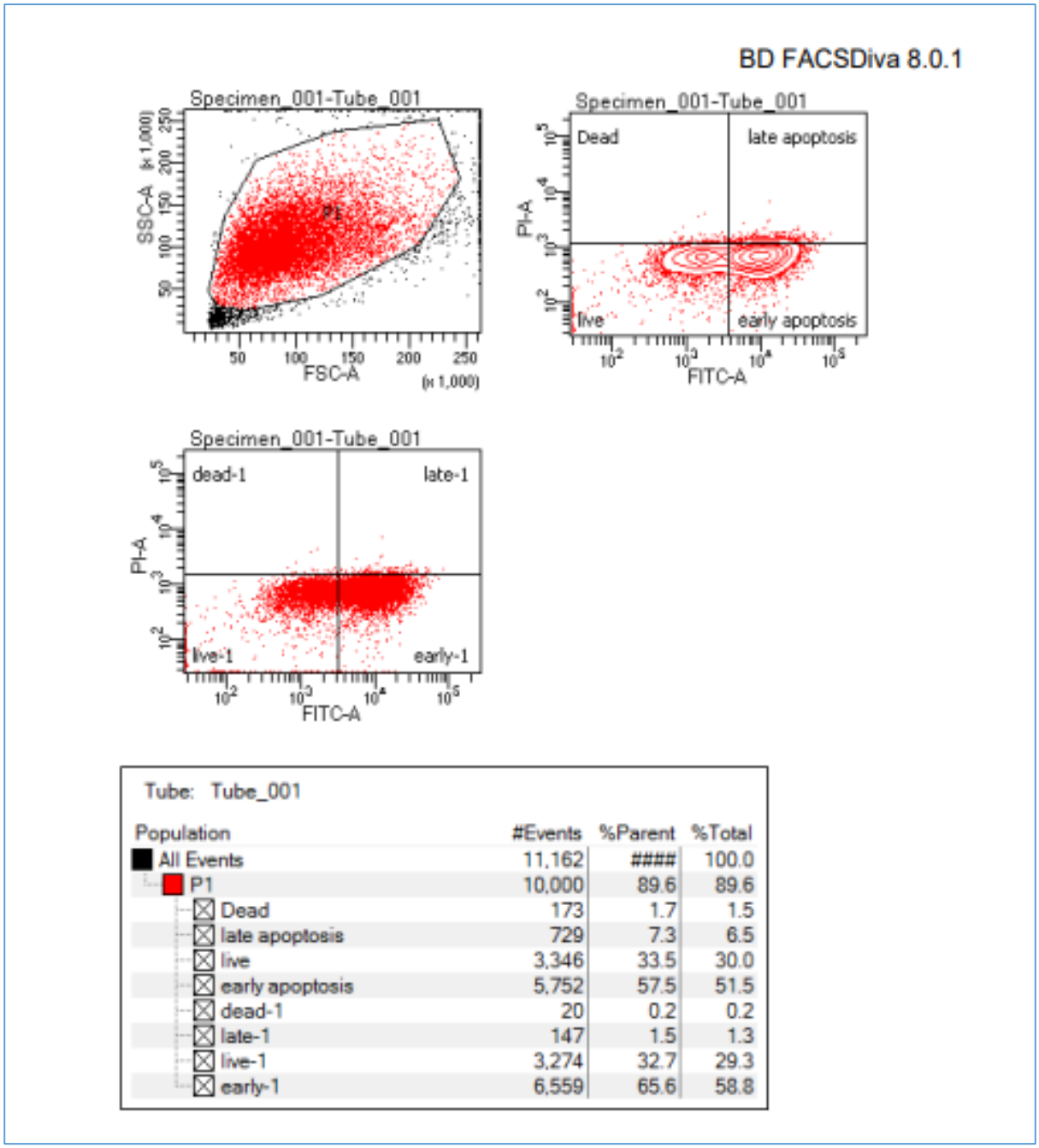
Fig. 7.9. Clone formation analysis of Lactobacillus strain A) untreated cell line, B) *L. rhamnosus* C) *L. plantarum* and D) *L. acidophilus*

All the results are compared with the control group (Fig. 7.9. A). A similar experiment was performed by using soybean *L. plantarum DGK* fermented extract at a concentration of 100 µg showing the significant decrease of clone formation as compared with the standard oxaliplatin (24 µM) [7]. It is concluded that *L. plantarum* shows a cytotoxic effect on the colon cancer cell line HCT 115 as compared with other used LAB reference strains.

7.3.6 Annexin V/PI analysis of Lp, Lr, and La

HCT 115 cells are treated with the supernatant of Lp, Lr, and La for 24 h. Thus, mixed results of the fluorescent dots (annexin V^+/PI^-) are observed in the lower right quadrant indicating the early apoptosis phenomenon in case of Lp, and

Lr, while no any such dots are observed in case of the reference standard *L. acidophilus*. Similarly mixed results of the fluorescent dots (annexin V^+/PI^-) are observed in the upper right quadrant indicating the late apoptosis phenomenon in cases of Lp, and Lr, while no any such fluorescent dots are observed in case of the reference standard La. The results revealed that about 57.5% of the early and 7.3% late apoptosis are observed on the HCT 115 cell lines by treatment with the Lp supernatant (Fig. 7.10. A). In the case of the Lr, the early and late apoptosis observed on the HCT 115 cell line was about 22.2% and 6.0% respectively (Fig. 7.10. B). The results also revealed that 0.0% and 0.1% of the early and late apoptosis are observed on the HCT 115 cell lines by treatment with the La supernatant, which was used as the reference standard control group in the study (Fig. 7.10. C). The necrotic dead count observed in the upper left quadrant in the case of Lp, Lr, and La were 1.7%, 6.4% and 0.7% respectively which are not affecting the overall study which was conducted. The pro-inflammatory agents are released due to the death of the cells by the necrosis phenomenon, while the death of the cells by the apoptosis is the cascade events. It concludes that the death in the cell lines is due to the phagocytosis phenomenon, induced by the macrophages and cytokines due to the complementary system activation. The PI inclusion in assay assesses the membrane integrity during the apoptosis process [10].



Tube: Tube_001

Population	#Events	%Parent	%Total
All Events	11,162	####	100.0
P1	10,000	89.6	89.6
Dead	173	1.7	1.5
late apoptosis	729	7.3	6.5
live	3,346	33.5	30.0
early apoptosis	5,752	57.5	51.5
dead-1	20	0.2	0.2
late-1	147	1.5	1.3
live-1	3,274	32.7	29.3
early-1	6,559	65.6	58.8

Fig. 7.10. A. Annexin V analysis of *L. plantarum*

While the staining with the FITC- conjugated annexin V differentiates the category of the death as necrosis or apoptosis (early or late). The current study proves that the Lp exhibit as early and late apoptosis as compared with the Lr with negligible necrosis events.

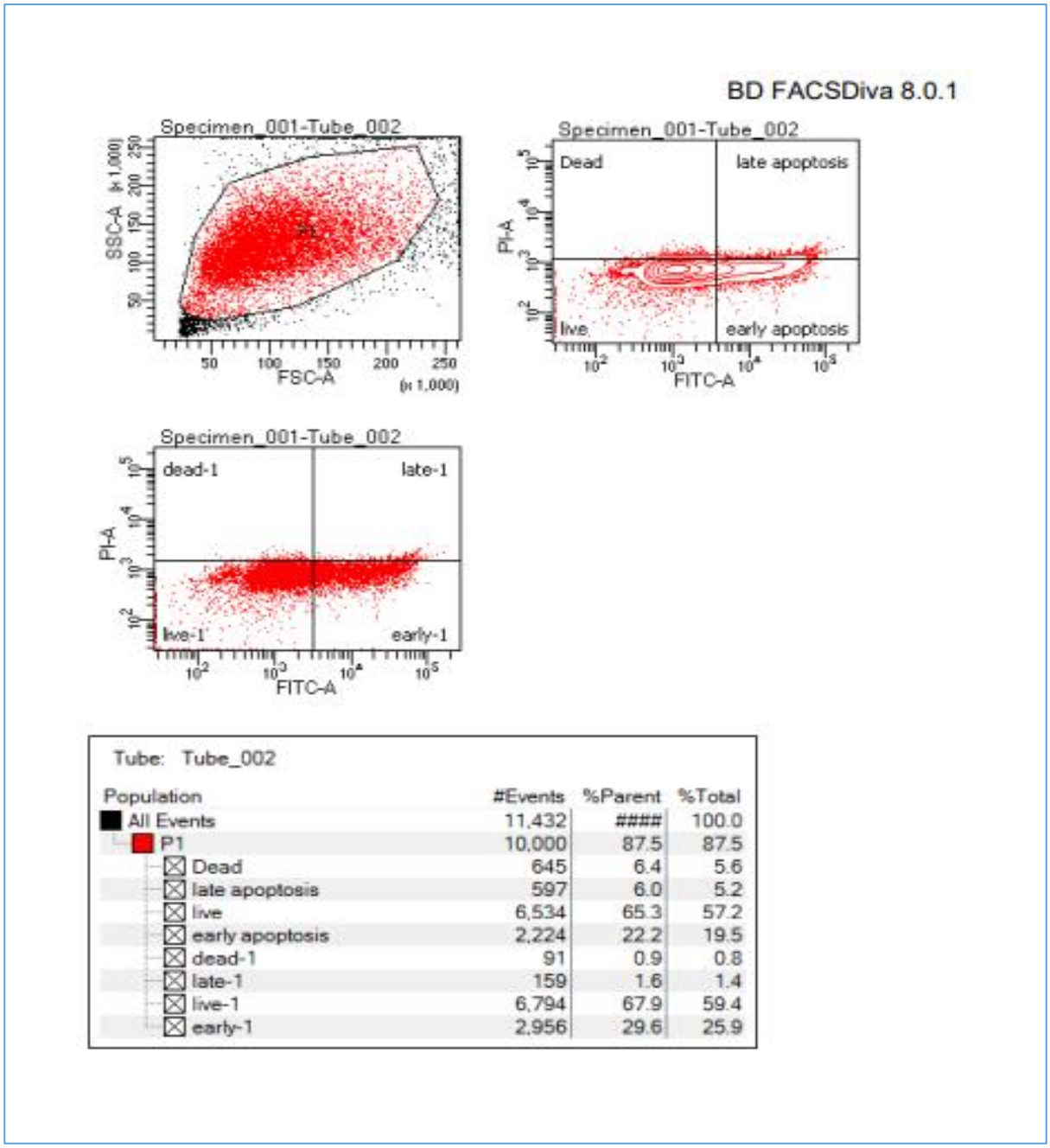
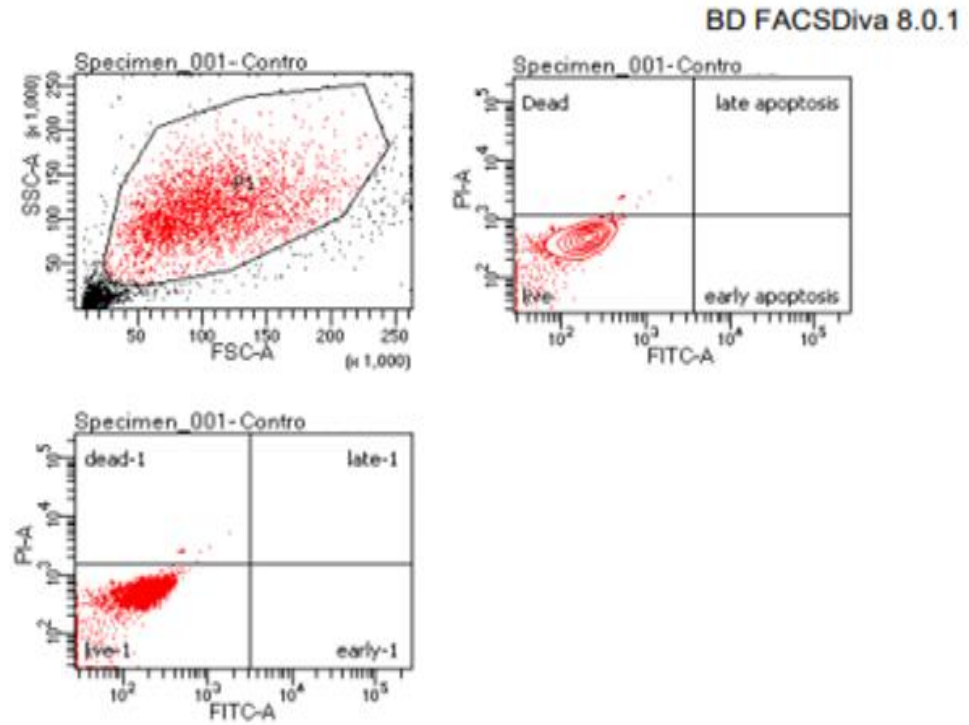


Fig. 7.10. B. Annexin V analysis of *L. rhamnosus*

The earlier studies have proven that *L. plantarum SBL* with the concentration of about 50 µg/ml after 24 h showed 20% and 51.5% early and late apoptosis on Hela cell lines [10].



Tube: Unstained Control

Population	#Events	%Parent	%Total
All Events	5,000	####	100.0
P1	2,983	59.7	59.7
Dead	22	0.7	0.4
late apoptosis	0	0.0	0.0
live	2,961	99.3	59.2
early apoptosis	0	0.0	0.0
dead-1	11	0.4	0.2
late-1	0	0.0	0.0
live-1	2,972	99.6	59.4
early-1	0	0.0	0.0

Fig. 7.10. C. Annexin V analysis of *L. acidophilus*

7.3.7 Up/down-regulation of the apoptotic protein expression of Lp, Lr, and La

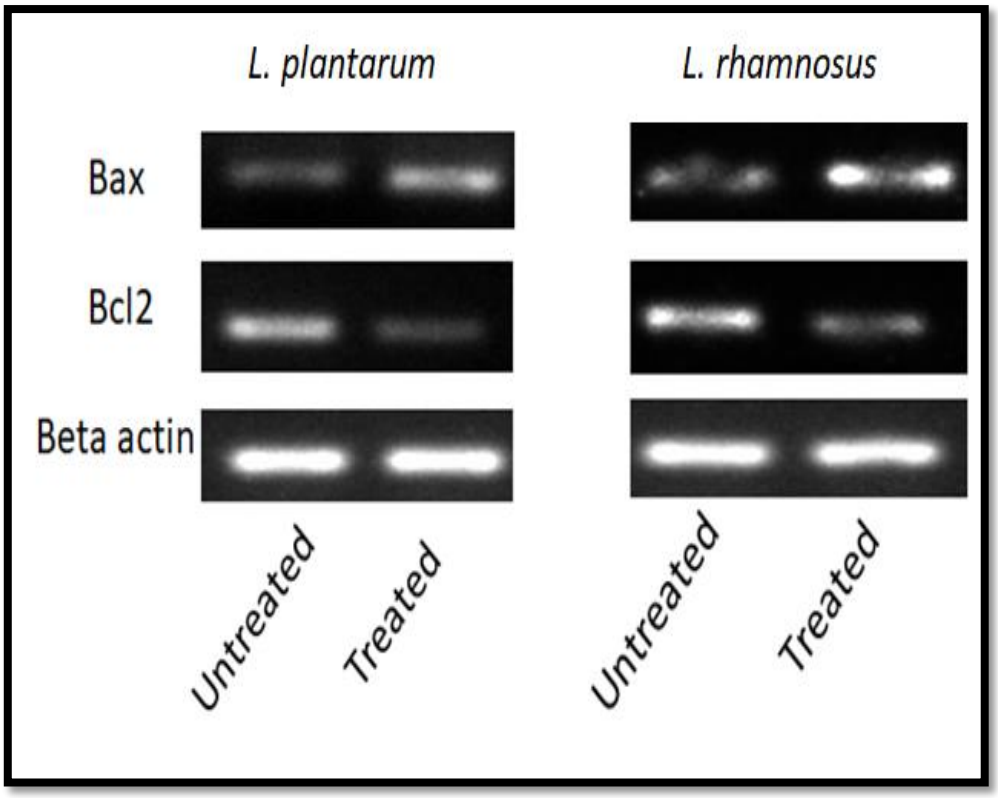


Fig. 7.11. Apoptotic protein expression by the Lactobacillus strain

The apoptotic proteins were investigated by the up-regulation and down-regulation of the Bax, Bcl2 and beta-actin along with its expression ratio. Bax acts as pro-apoptotic proteins, it shows a significant increase (p<0.01) in the Bax of Lp (treated) compared with the (untreated) control group. Similarly, there is a significant increase (p<0.05) in the Bax of Lr compared with the control group. Thus increase in Bax is found higher in Lp as compared to Lr (Fig. 7.11). Similarly Bcl2 acts as anti-apoptotic proteins, it shows a significant decrease (p<0.01) in the Bcl2 of Lp compared with the control group. Similarly, there is a significant decrease (p<0.05) in the Bcl2 of Lr compared with the control group. Thus, a decrease in Bcl2 expression is seen higher in Lp as compared to Lr. No, any significant changes are observed in the Beta-actin expression in case of Lp and Lr for both treated and untreated groups. This shows that Lp shows significantly higher apoptotic protein

expression properties as compared to Lr. Apoptotic activities were reported by *L. reuteri* on HT 29 colon cancer cell lines by an increase in Bax and decrease in Bacl2 [16]. In a similar way, *L. plantarum* shows apoptotic activity by up-regulation of Bax and down-regulation of Bcl2.

7.3.8 Adhesion ability of Lactobacillus on the cell line by SEM analysis

The Fig. 7.12 (A) represent control group without any microbial inoculation, while in fig 7.12 (B) HCT 115 cells are inoculated with Lr showing cell adhesion at a minimal level. On the contrary, Lp shows significantly higher adhesion to HCT 115 cell line which as seen in the fig 7.12 (C). While no any kind of cell adhesion is observed in the case of La as seen in fig 7.12 (D). Previous studies showed that *P. acidilactici* MTCC5101 showing stronger adhesion to intestinal epithelium Caco 2 cells of the mucosal area [17].

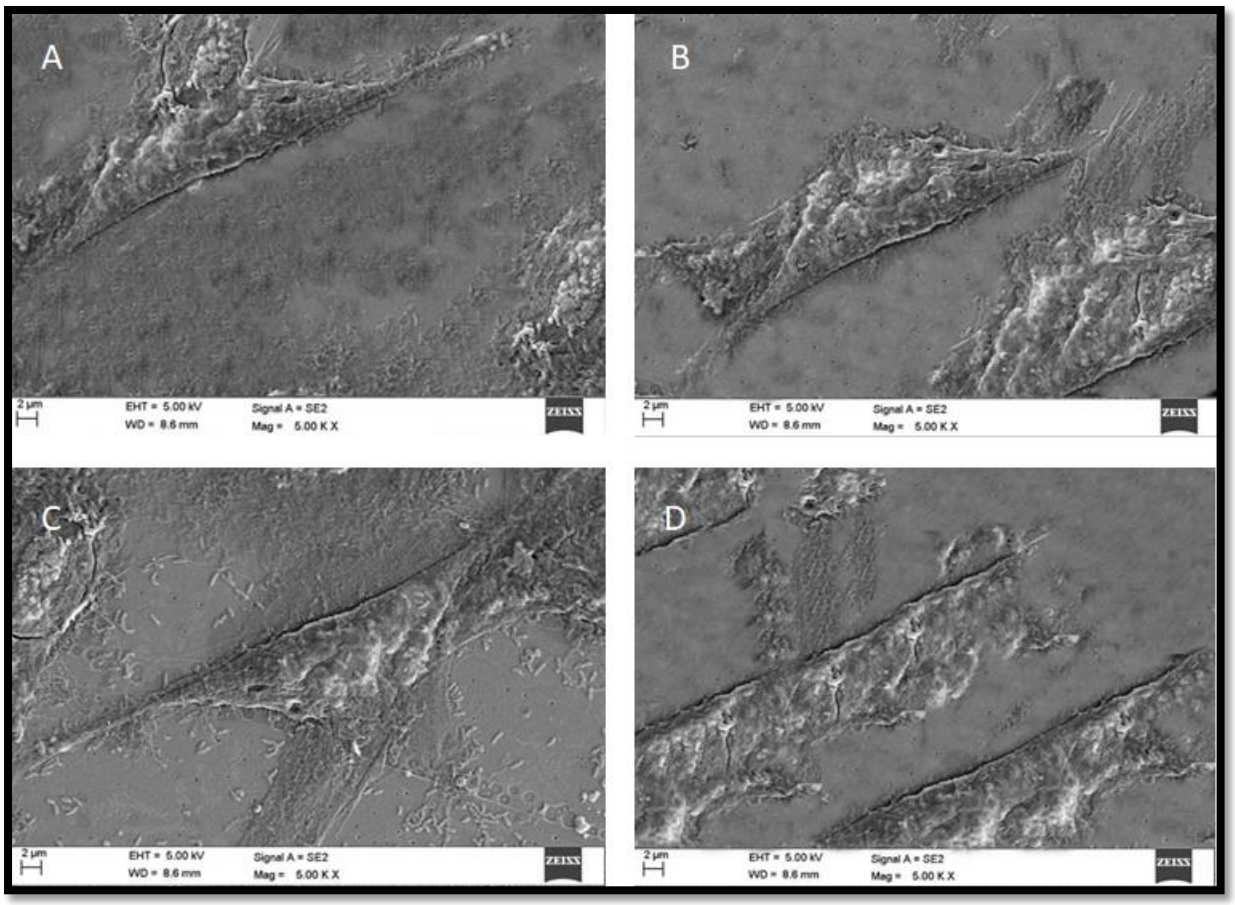


Fig. 7.12. SEM adhesion assay of lactobacillus A) Control, B) *L. rhamnosus*, C) *L. plantarum* and D) *L. acidophilus*

7.4 Conclusions

The present study deals with the comparative cytotoxicity studies of Lactobacillus against the cancerous cell lines. The study shows that *L. plantarum* acts as an effective antiproliferative agent against cancer cell lines as compared to the *L. rhamnosus* and *L. acidophilus. L. plantarum* shows cell death by the apoptosis due to nuclear fragmentations. The current study shows that the *L. plantarum* exhibit the cell death mechanism as early and late apoptosis as compared with the *L. rhamnosus* with negligible necrosis events. The apoptotic protein expression studies show that *L. plantarum* kills the cancerous cell by up-regulation of Bax and down-regulation of Bcl2. The cell adhesion SEM study reveals the binding tendency of the *L. plantarum* to cancer cell lines by stronger adhesive forces. Thus, the *in-vitro* study shows the cancer killing tendency of the *L. plantarum* is by the combination of antiproliferative, adhesion and gene-protein regulation mechanisms.

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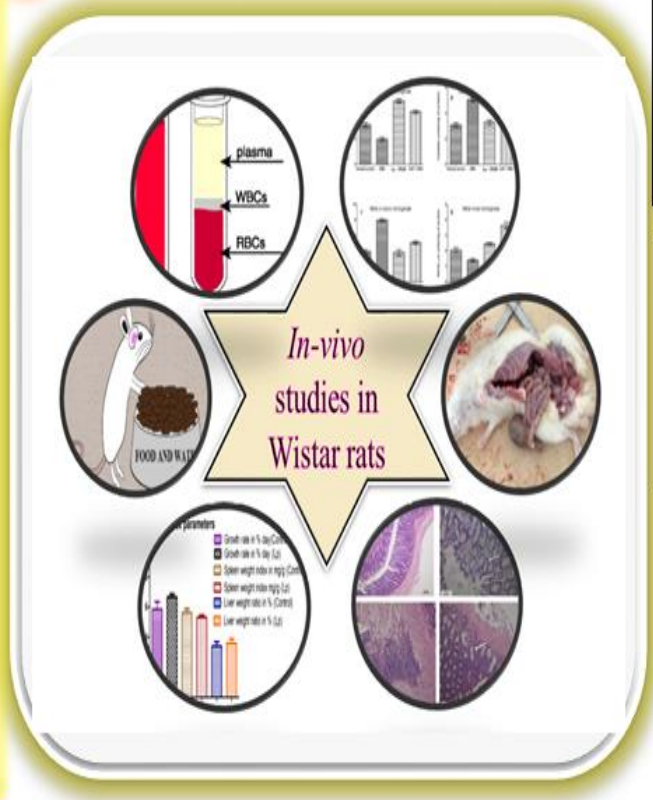
Chapter 8

The part of this chapter have been published as a research article

In-vivo Antioxidant and Anti-colon cancer activity of Lp formulation



SMGroup
International Journal of Animal Science
Short Communication

Probiotics with Dimethyl Hydrazine Induced Animal Study as New Psychological Study Model

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Rapid Communication

Lactobacillus Acidophilus as a Preferable Natural Anticancer Agent

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RESEARCH ARTICLE

Probiotic Potential of *Lactobacillus Plantarum* with the Cell Adhesion Properties

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8.1 Introduction

Functional foods nowadays act as a biological and therapeutical agent which when incorporated into the diet as nourishment, proposed to reduce the risk of various disease [1]. The oxidative damage to the cell and/ or its components mostly results in dreadful diseases such as cancers, degenerative and cardiovascular diseases [2]. Probiotics and there various product utilities such as 'yogurt, dahi' is supposed to relieve the host cells from free radicals generation, without causing any adverse effects. The synthetic antioxidant drug moieties available in the market, are found with chronic side effects triggering liver damage and induction of carcinogenesis [3]

The probiotics include many different bacterial strains, each of these species differs in nature and activity [4]. Most of the times strains of the same species are unique in characteristics such as adherence, immunological activity and other biological actions on the host [5]. Currently, probiotic research aims in the isolation of new strains and investigation of microbiota and microbiome characteristics in each part of the gastrointestinal tract from the small intestine to the colonic region [6]. The basic focus of research includes the host-microbe interactions within the intestine, microbe-pathogen interactions within the microbiota and the microbe-drug interactions in diseased conditions. The health benefits observed due to the uses of probiotics is mediated by means its antioxidant activity [7]. The antioxidant mechanisms of probiotics could be due to metal ion chelation, enzyme inhibition and free radical scavenging mechanism [8].

Dimethylhydrazine (DMH) is used to enhance the oxidative stress conditions in an animal models [9]. Mostly DMH is used to induce cancer in a Wistar rat model. The mechanism involved is the induction of severe oxidative stress conditions in the host tissue or organ system hindering the cellular activities [10]. Capecitabine (CAP) is used as the standard synthetic drug to heal chemical-induced cancer by DMH, which is available as a medicine in the global market [11]. But this synthetic drug is

having innumerable side effects such as an imbalance in the oxidative stress level in the cell and damage to the enzyme system of the liver in the host [12].

In the current study, the *L. plantarum JDARSH* MCC 3595 (Lp) isolated from sheep milk is investigated for various pharmacological activities. We evaluated various probiotics characteristics properties in previous studies and showed Lp as pH, temperature, bile salt, and NaCl tolerant [13]. Considering all these facts, further investigations and an attempt of its first kind are made to evaluate the antioxidant mediated anti-colon cancer potential of probiotics Lp. The study focuses on comparing the Lp with the standard drug capecitabine by *in-vivo* DMH induced oxidative stress rat model.

8.2 Experimental

8.2.1 *In-vivo* animal study

The antioxidant and anti-colon cancer animal study model was approved by the IAEC; TKCP/2015/13, Warananagar, India. In order to monitor toxicity, the study was divided into two parts as a) oral acute toxicity study and b) anti-cancer activity. Animals were kept in stainless steel cages covered with sterile paddy husk as per the specification is given by IAEC. For oral acute toxicity study, the animal was categorized into two treatment groups designated as control A and test B group (3 Wistar rats in each group). F4 spray dried formulation due to high viability count was selected for *in-vivo* studies. In order to study the toxicity profile of Lp, a single dose of 5000 mg/Kg was administered to test group by per oral route to each of the rat using feeding needles by dosing single animal at a time. The study was conducted as per the guidelines provided by OECD (Organisation for economic co-operation and development). Rats of the control group received the sterile saline solution containing 5% of dextrose. Animals were monitored for about 14-15 days regularly to see any changes in activities, behavior and general health condition along with its weight. In order to re-evaluate the results, group B (test) were again re-experimented with the dose of 5000 mg/Kg (3 animals). Simultaneously, the feces of the rat were collected time to time on day 0, 3, 6, 9 12 and 15 to enumerate the total Lactobacillus

count using MRS media and the level of enterobacteria was estimated by growing them in MacConkey agar.

Table 8.1. MacConkey agar media compositions

Media ingredients	Formula (%)
Peptone	17 g
Proteose peptone	3 g
Lactose	10 g
Bile salt	1.5 g
Sodium chloride	5 g
Crystal violet	0.001 g
Agar	13.5 g
Water	1 litre
Final pH (at 25 °C) Agar pH adjusted to 6.8 at 25 °C	

Haematological studies of both (control) A and (test) B group were carried out with other vital organ analysis to determine the aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) contents. Similarly, liver marker estimation and different body index parameters such as the weight of liver and spleen of both animal groups were determined.

After successful completion of acute oral toxicity study, anti-colon cancer study was carried out. While, for conducting the anti-colon cancer activity, twenty-four 6 weeks older male Wistar rats having weight in the range of 150-200 g were

obtained from the central animal house Warananagar. Animals were acclimatized in stainless steel cages padded daily with sterile paddy husk at controlled room temperature 24 ± 3 ºC and humidity 55 ± 4% at 12:12 h of light: dark cycles [14]. The study was conducted on Wistar rats (n=24), where four groups were assigned: vehicle control (n=6), DMH treated group (n=6), Lp + DMH group (n=6) and CAP + DMH group (n=6). The vehicle used for the preparation of dose is saline water [10]. Animals were fed with rodent laboratory diets (Pranav Agro Ltd. Sangli) with a frequent supply of drinking water. Animals are fasted for 12 h, prior to dosing (no food but water is not withheld). The fasted body weight of all animals in each group was determined from time to time. The dose given to all animals were calculated according to their body weights.

The rats from all groups except vehicle control received DMH at 20 mg/kg body weight (b.w.) by the subcutaneous (S.C.) route, dissolved in 1mM EDTA once a week for a period of 6 weeks. This DMH act as cancer inducer by increasing the cellular oxidative stress level in colonic cells. The rats from the Lp + DMH group received 500 mg/kg/day dose of Lp, by the per-oral route. The rats from the DMH group received only DMH which was purchased from Sigma-Aldrich, USA. The animals from CAP + DMH groups received capecitabine 150 mg/kg/day by the intravenous route, which was received as a gift sample from the Department of Pharmaceutics, TKCP, Warananagar, India. The results of the CAP + DMH group was considered as a standard group so was compared with the Lp + DMH group. A vehicle control group received saline water by the per-oral route and acts as a placebo. Later, the animals were screened for the weekly measurement of body weight up to 6 weeks. Daily measurement of food and water consumption is done up to 6 weeks with an analysis of hematological status on the last day before euthanasia. Approximately 5 ml of intracardiac blood were removed from rats under the sodium pentobarbital anesthesia.

8.2.2 Oxidative stress parameters measured in DMH induced Wistar rat's model

After completion of the study, animals were sacrificed by cervical dislocation method by prior treatment with a lethal dose of sodium pentobarbital (50 mg/ml). Later, the colonic parts, spleen, and liver of rats were dissected; subjected to saline transcardially in ice-cold condition for its extraction [15].

The colonic pieces of 8 cm were fixed using 10% buffered formalin on filter paper and stained with help of 0.1% methylene blue stain in PBS for 15 min. The aberrant crypt foci (ACF) and ACF counts were determined at 40X resolution using a light microscope. The ACF counts were calculated as the number of ACF per 5 cm^2 denoted as Φ, while the number of ACF per cm^2 was denoted as Ω. Histopathological examinations were determined by using hematoxylin-eosin reagent. Hemoglobin (Hb), white blood cell count (WBC), red blood cell count (RBC) were measured using a cell counter (Celltac- NIHON KOHDEN, Germany). The lymphocyte and neutrophil percentage were counted using the optical microscope. The liver and spleen were removed from all treated groups, washed with PBS solution. The liver and spleen indices were calculated by dividing the weight of the individual organ with the weight of the whole animal [16].

Catalase activity was determined from the extracted tissues of the colon and liver. The fragmented tissues were homogenized using PRO 200 homogenizer (PRO scientific, USA) for 40 s at 6,000 x *g* centrifugation using 0.5% triton X-100 (Sigma Aldrich, USA). The catalase activities were evaluated using ELISA kit (CAT EC.1.11.1.9. RAYBIOTECH, USA) based on the method described by Johansson et al. [17] with modification. Absorbance was measured at 500 nm wavelength. The readings were taken by using formaldehyde as standard at 20 ^oC with a value of 6.8% coefficient of variation. Similarly, the same homogenized tissue samples were used to determine the nitrite contents of liver and colonic samples from all treated animal groups. The nitrite contents of tissue were investigated by taking 100 µl of tissue homogenate incubated with (20 mmol/l) of Tris, NADPH (80 µmol/l) and

nitrate reductase (50 µmol/ 100 µl sample) as per the method described by Guevara et al. [18] with slight modification. The final absorbance for the samples was measured at 540 nm using a microplate reader (LABSYSTEMS, USA).

8.3 Results and Discussion

8.3.1 Oral acute toxicity study and fecal matter analysis of Lp formulation

No any single death of an animal is reported during the whole study in both control (A) and test (B) group. Similarly, no mortality is reported in case of re-experimented test (B) group, which is carried out for re-validation of test results. The health status is observed in the cases of rats fed with Lp, which not shows any significant difference with the control group in terms of growth rate. Lp fecal matter contents in log cfu count are observed significantly ($p<0.01$) higher in the treated group as compared to the control group (Fig 8.1). In the control group, log cfu value of Enterobacteria and Lp are nearly the same. While, in the case of the test group, log cfu value of Lp increases significantly with a decrease in the level of Enterobacteria.

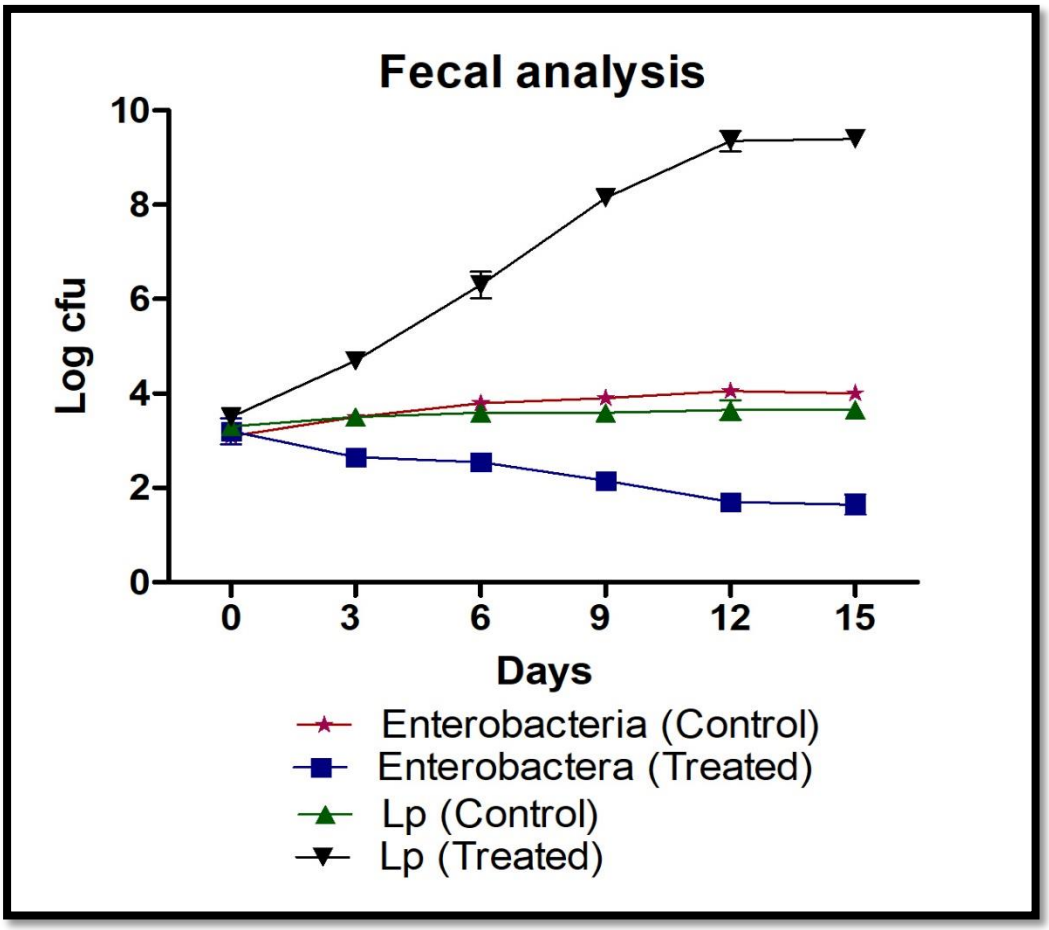

Fig 8.1. Fecal matter analysis of the Wistar rats model

These results are evaluated for the period of 15 days of studies at a regular interval of 3 days. Liver markers such as AST level observed in case of Lp treated group are found significantly higher ($p<0.01$) as compared to control group (Fig 8.2).

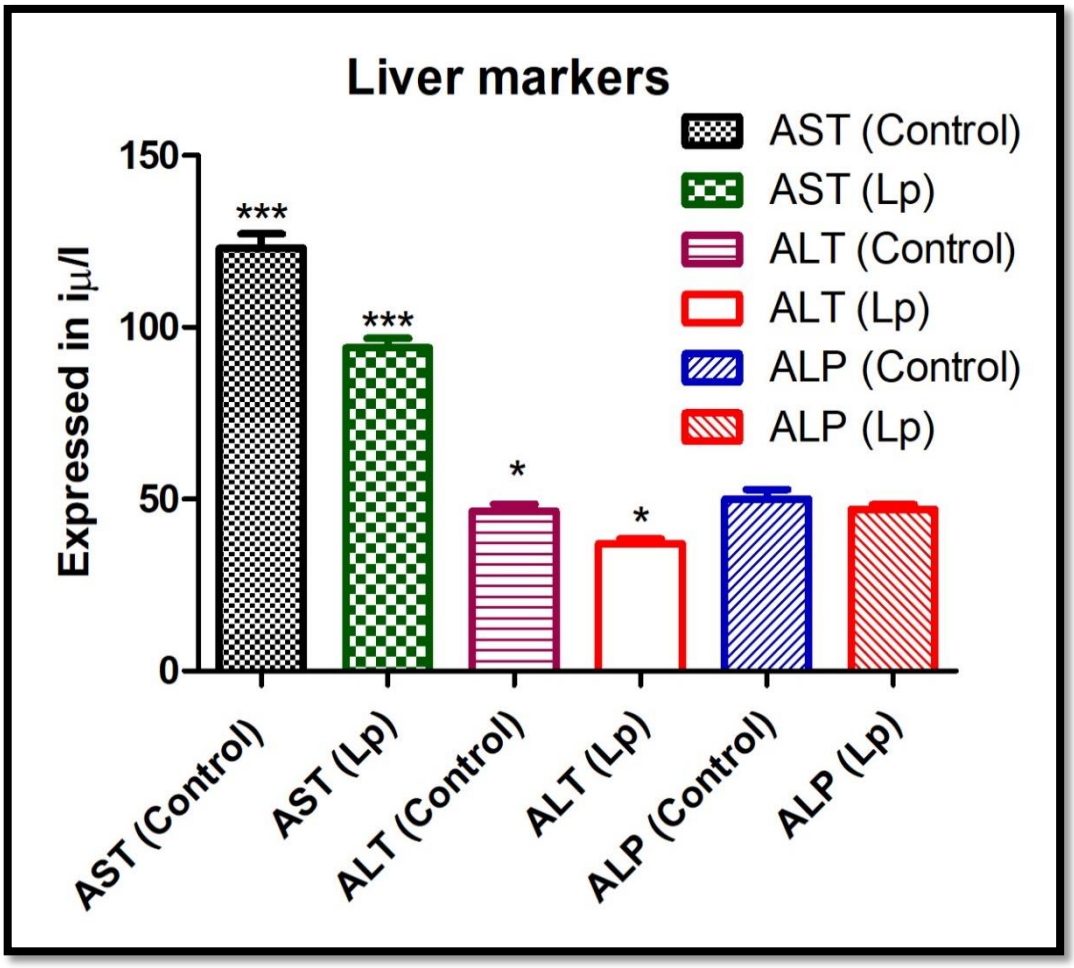

Fig.8.2. Analysis of the effect of Lp on liver markers compared with the control group

While the ALT level observed in case of Lp treated group are statistically significant (p<0.05) as compared to control group (Fig 8.2).

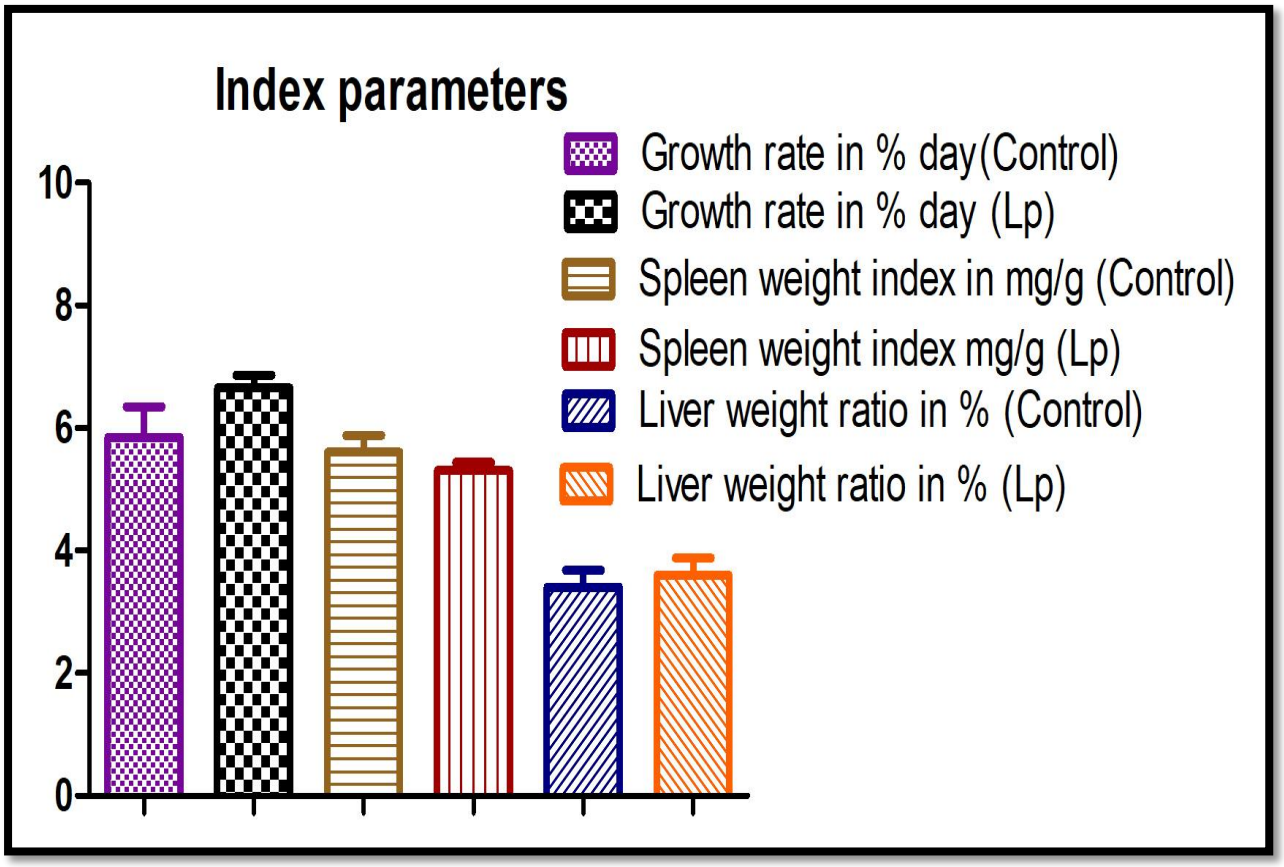

Fig 8.3. Analysis of the effect of Lp on different index parameters compared with the control group

No, any significant differences were observed in both groups in the case of ALP values (Fig 8.2). The index parameters such as the liver weight ratio and spleen weight index observed in the case of both group show no significant differences (Fig 8.3). No, any diarrhoeal mortality was observed in the cases of Lp treated and control groups. This shows that the Lp is inhabiting in all parts of large intestine including the colon. No, any significant differences are observed in the cases of both treated and untreated groups in term of percentage growth rate, liver weight ratios, and spleen weight index. This indicates that the Lp is non-invasive and not precipitate any systemic infection in the host.

Thus it shows that *L. plantarum* is non-toxic as it did not precipitate the toxicity in the animals and no any diarrhoeal death were reported. The liver marker

studies show that no any kind of hepatotoxicity reported in the host suggesting *L. plantarum* as non-toxic nutraceutical's agent.

8.3.2 *In-vivo* pharmacological analysis of Lp formulation

No, any single mortality is reported in animals during the whole study. The physiological state of the animal after drug treatment was identified by its behavior pattern; mainly by the food and water intake analysis. This investigational data gives the basic idea about the responses of an animal to the given treatment with the determination of the drug-induced pharmacological activities. The effect of treatments on food intake of animals are observed for 6 weeks and it shows a significant difference between all groups (Table 8.2). The Lp + DMH group [12.05 ± 0.48 (g/rat/day)] shows a significant ($p<0.05$) increase in food intake as compared to DMH treated group [10.48 ± 0.38 (g/rat/day)], CAP + DMH group [10.25 ± 0.53 (g/rat/day)] and shows nearly same values as that of vehicle control group [13.15 ± 0.41 (g/rat/day)]. This reflects that DMH not affects the physiological metabolism of Lp treated group; further, the values found were the same as that of the vehicle control group. It also shows that Lp not only maintained the proper bowel condition of the host but also protected it from oxidative stress-inducing chemicals like DMH. Reduction in food intake is observed in DMH treated animal. The reason is overexpression of transthyretin which acts as a blood transporter, modulating the energy balance and decreasing the food intake [19]. In the current study, it is found that Lp helps to maintain the bowel movement of the gastrointestinal tract, may be by preventing the overexpression of transthyretin responsible for the loss of appetite. Probiotics are found helpful in digestion and proper functioning of the gastrointestinal tract in the host, which was demonstrated by many previous studies [20].

Additionally, the intake of water in all treated groups was observed from time to time. Water intake observed in case of Lp + DMH treated group [18.88 ± 1.32 (ml/rat/day)] are nearly same as that of vehicle control group [18.02 ± 1.16 (ml/rat/day)]. On contrary, water intake is observed increased significantly ($p<0.05$) in cases of DMH treated animals [19.17 ± 0.92 (ml/rat/day)] and CAP + DMH group

[19.32 ± 1.34 (ml/rat/day)] (Table 8.2). It implies that the dose of Lp maintains the normal basal metabolism of animals as the vehicle control group and which is not seen in synthetic drug capecitabine.

Table 8.2. Effects of treatments on daily consumption of food and water

Groups/ treatment	Food consumption (g/rat/day)	Water consumption (ml/rat/day)
Vehicle control	13.15 ± 0.41	18.02 ± 1.16
DMH treated	10.48 ± 0.34*	19.17 ± 0.92*
Lp + DMH	12.05 ± 0.44*	18.08 ± 1.32*
CAP + DMH	10.25 ± 0.54*	19.32 ± 1.34*

Values are expressed as mean ± *SEM* *= $p<0.05$

The body weight of the Wistar rats is observed periodically for a period of 6 weeks (Table 8.3). The final animal body weight is compared to their initial body weight for all studied groups. Lp + DMH treated group shows 60 g (approximately) significant ($p<0.01$) increase in body weight as compared to the CAP + DMH group showing 17 g weight gain, which is highly significant. The increase of body weight of the vehicle control group 60 g observed is nearly the same as Lp + DMH treated group. This indicates that the weight gain using Lp is higher than using the capecitabine during the whole study protocol. The sluggish weight gain in CAP + DMH group is due to loss of appetite in animals and can be considered as a side effect of this synthetic drug.

Table 8.3. Effects of treatment on body weight of DMH induced rats

Treatment	Initial weight (g)	Final weight (g)	Body weight gain (g) (approximately)
Vehicle control	190.0 ± 4.47	250.0 ± 4.47	60
DMH treated	193.5 ± 4.21*	196.0 ± 4.42*	2.5
Lp + DMH	193.3 ± 3.70**	240.2 ± 4.04**	47
CAP + DMH	193.3 ± 4.21**	210.3 ± 3.46**	17

Values are expressed as mean ± *SEM* *= $p<0.05$, **= $p<0.01$

8.3.3 Evaluation of hematological parameters of Lp formulation

There is marked decrease in Hb [8.85 ± 0.66 (g/dl)] of DMH treated animals as compared to vehicle control group [12.16 ± 0.30 (g/dl)] of Wistar rats. The significant ($p<0.01$) increase in the Hb value of Lp + DMH group [11.98 ± 0.42 (g/dl)] is observed as compared to CAP + DMH group [10.83 ± 0.47 (g/dl)], which is used as a standard synthetic drug for treatment (Table 8.4).

Table 8.4. Effects of treatment on Hb/ RBC/ WBC count of DMH induced rats

Group	Hb (g/dl)	(RBC) X 10^6/mm^3	(WBC) /mm^3
Vehicle control	12.16 ± 0.30	7.75 ± 0.13	6325 ± 141.27
DMH treated	08.85 ± 0.66*	5.60 ± 0.16*	13889 ± 144.72*
Lp + DMH	11.98 ± 0.42**	7.10 ± 0.17**	6516 ± 087.22**
CAP + DMH	10.83 ± 0.47**	6.08 ± 0.13**	8916 ± 094.57**

Values are expressed as mean ± *SEM* *= $p<0.05$, **= $p<0.01$

The effects of treatments on hematological parameters are observed which shows that DMH treated animals with a decrease in the RBC count [5.60 ± 0.16 X 10^6/ mm^3] as compared to the vehicle control group [7.75 ± 0.13 X 10^6/ mm^3] (Table 8.5). On contrary, the RBC count significantly ($p<0.01$) increased in Lp + DMH group [7.10 ± 0.17 X 10^6/ mm^3] as compared to CAP + DMH group [6.08 ± 0.13 X 10^6/ mm^3]. The level of WBC is found to be markedly increased in DMH treated [13889 ± 144.72/ mm^3] animals as compared to vehicle control group [6325 ± 141.27/ mm^3] (Table 8.5). There is significant decline ($p<0.01$) in WBC count of Lp + DMH group [6516 ± 087.22/ mm^3] as compared to CAP + DMH group [8916 ± 094.57/ mm^3], but it is same as that of the vehicle control group. The hematological evidence showed that the Hb level is decreased with an increase in WBC count by DMH, which may further lead to cancer or even another dreadful disease [2]. The decrease in Hb level results in anemia and is found to be greatly affecting the pathophysiological conditions of cancer patients [21]. Many previous studies have proven that DMH not only affects the formation of hemoglobin or RBC but also alter the hemopoiesis pathway [24]. This indicates Lp corrects the hemopoiesis process affected drastically due to DMH treatment which is not seen in the capecitabine-

treated group. The decrease in Hb value during DMH treatment is an indication of an increase in oxidative stress [25].

Table 8.5. Differential Leukocyte Count (%)

Group	Differential Leukocyte Count (%)	
	(Neutrophils) X 10^2/ mm^3	**(Lymphocyte)** X 10^2/ mm^3
Vehicle control	32.2 ± 0.73	62 ± 1.95
DMH treated	38.2 ± 0.84*	38 ± 1.66*
Lp + DMH	31.5 ± 0.42**	58 ± 1.52**
CAP + DMH	33.6 ± 0.42**	42 ± 1.52**

Values are expressed as mean ± *SEM* *= $p<0.05$, **$p<0.01$

The level of neutrophil is found to be significantly increased in DMH treated group [38.2 ± 0.84 X 10^2/ mm^3] as compared to the vehicle control group [32.2 ± 0.73 X 10^2/ mm^3] which is showing less value (Table 8.5). On contrary the neutrophil count found in Lp + DMH group [31.5 ± 0.42 X 10^2/ mm^3] is significantly ($p<0.01$) decreased as compared to the CAP + DMH group [33.6 ± 0.42 X 10^2/ mm^3]. This describes that Lp dose does not trigger the WBC activation, while the capecitabine-treated group shows higher WBC activation. Thus, activation of WBC is regarded as the false alarming inside the host in the absence of disease condition [11].

Similarly, the lymphocyte count found in the case of DMH group [38 ± 1.66 X 10^2/ mm^3] is significantly ($p<0.05$) lesser as compared to the vehicle control group [62 ± 1.95 X 10^2/ mm^3] which is showing a higher value. The significant ($p<0.01$) increase in the lymphocyte value of Lp + DMH group [58 ± 1.52 X 10^2/ mm^3] is observed as compared to CAP + DMH group [42 ± 1.52 X 10^2/ mm^3]. Many

previous studies showed that the lowering of the Hb may be a sign of cancer [22]. The present study shows that Lp not only increased the Hb level but maintained neutrophils and lymphocyte count nearly the same as a vehicle control group. These results are in accordance with the previously reported studies describing the oxidative cellular damage by DMH and its prevention [6]. The administration of DMH is found associated with the increase in the WBC count and altering the microenvironment of the tissue [23].

In the present study, the administration of Lp shows a decrease in the oxidative stress with the false activation of WBC and conditions like inflammation of the colon caused by DMH induction. As the DMH was found to increase the WBC count, it resulted in the triggering of colitis in mice which was corrected by functional food containing Vitamin E [26]. The current study demonstrates that the Lp may work in a similar pathway as Vitamin E in correcting the colonic damage which was caused due to DMH induction. Previous studies proved that lymphocyte count get reduced by induction of DMH in an animal model [27]. The present study shows that Lp increases the lymphocyte count by counteracting the effects of DMH as compared to the capecitabine-treated group.

The present study apportioned with growing and proper utility of *L. plantarum* as an antioxidant mediator. Lp acts as a natural antioxidant mediator, as it prevented the DMH elevated chemical-induced oxidative stress condition observed in Wistar rats. The cfu count of probiotics above 10^{7-8} is considered as a therapeutic concentration, responsible for showing the pharmacological activities [3].

8.3.4 Evaluation of enzyme and aberrant foci estimation of Lp formulation

The spleen indices of all animal groups do not show any significant ($p<0.05$) changes in their values (Fig. 8.4.A). Similarly, no significant differences are observed in liver indices of all treated groups (Fig. 8.4.B). This shows that DMH not affect liver cells but damage the colonic region. This indicates that Lp + DMH is not producing any splenomegaly condition. No any sign of hepatomegaly is observed in Lp treated group indicating normal liver functioning.

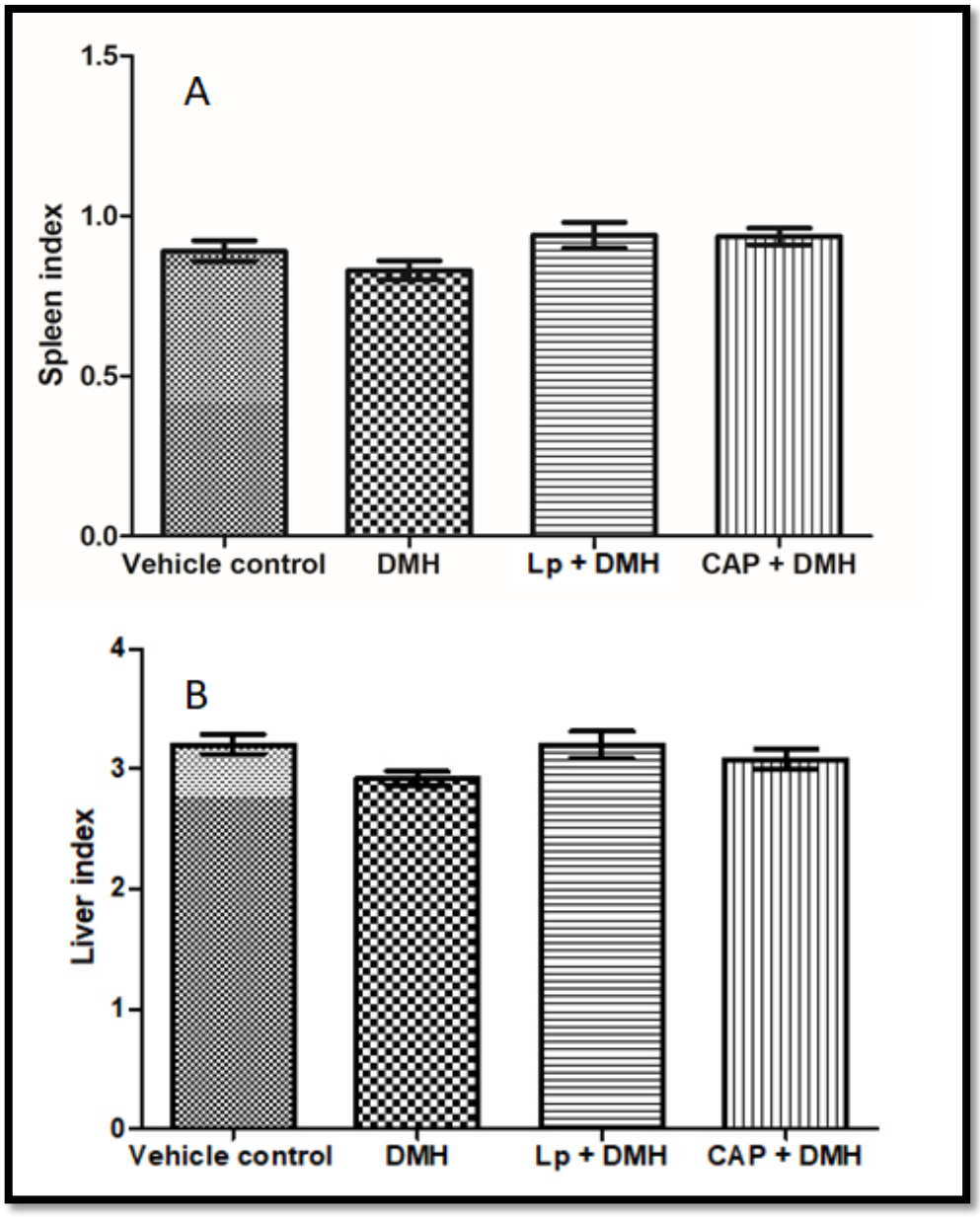

Fig 8.4. A) Spleen indices of the vehicle control, DMH, Lp + DMH, and CAP + DMH group & B) Liver indices of the vehicle control, DMH, Lp + DMH, and CAP + DMH group, data is represented as mean ± SEM of 5 values

Increase in size and numbers of the aberrant foci or ACF in the colonic tissue works as a biomarker in the determination of formed tumors. The present studies demonstrate that there are many structural changes in form of a larger number of ACF count in case of DMH treated group with $\Phi = 78.5 \pm 2.1$ (Fig. 8.5.A) and $\Omega = 18.2 \pm 1.2$ (Fig. 8.5.B) indicating the pre-carcinogenic conditions in the colonic

tissues. Lp + DMH group are found with $\Phi = 40.5 \pm 0.5$ (Fig. 8.5.A) and $\Omega = 8.2 \pm 0.6$ (Fig. 8.5.B) which shows significant ($p<0.05$) reduced ACF count formation as compared with CAP + DMH group with $\Phi = 48.5 \pm 0.3$ (Fig. 8.5.A) and $\Omega = 10.2 \pm 0.6$ values (Fig. 8.5.B). DMH generally undergo metabolism to give by-product as azoxymethane and its derivatives which are involved in the alkylation of colonic mucosal DNA. ACF formation is an indication of the alkylation of DNA of colonic mucosa [11]. This ACF is identified by methylene blue staining of the colonic region which acts as the precursor of adenoma and is observed in the case of DMH treated group [28]. The previous investigations reported that an increase in the oxidative stress in colonic mucosa exaggerate the ACF formations [10]. This shows that Lp scavenged the oxidative stress in cells, preventing ACF formation and is more DMH induced oxidative stress protectant than capecitabine.

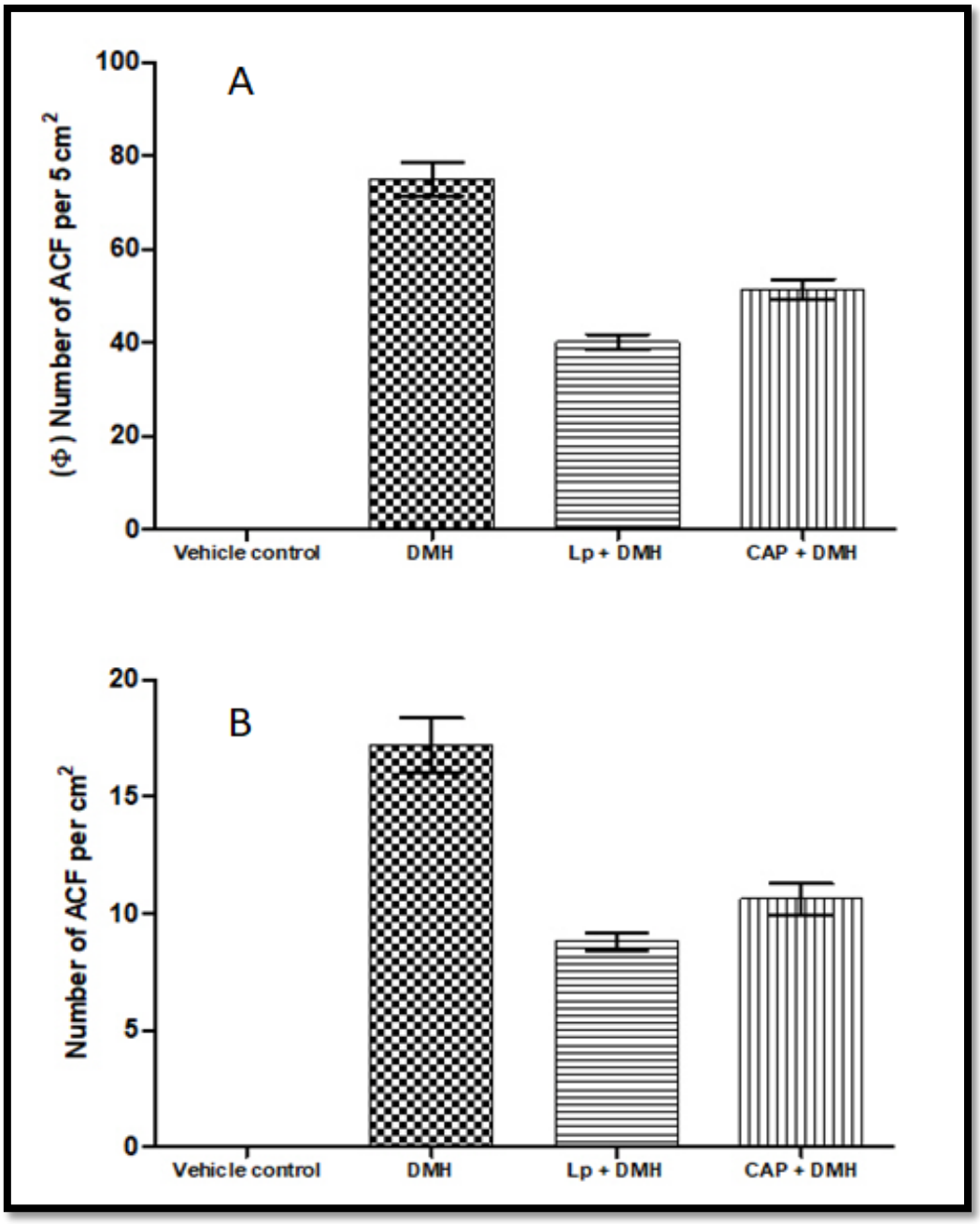

Fig 8.5. Aberrant crypt foci count A) (Φ) the number of ACF per 5 cm^2 & B) (Ω) the number of ACF per cm^2, in all groups. Data are represented as mean ± SEM of 5 values

The catalase and nitrite content in colonic and liver homogenate of treated groups are evaluated. Significant (p<0.05) elevation in colonic catalase content is observed in case of DMH treated group (5.2 ± 0.51 units/mg of protein), while no any comparative significant difference is observed in cases of Lp + DMH (3.4 ±

0.31 units/mg of protein) and CAP + DMH (3.4 ± 0.41 units/mg of protein) treated groups (Fig. 7.6.B). The Lp + DMH group (5.2 ± 0.42 units/mg of protein) shows a significant increase (p<0.05) in liver catalase content as compared to the CAP + DMH (4.1 ± 0.36 units/mg of protein) group (Fig. 8.6.A). On contrary, a significant reduction (p<0.05) in the colonic nitrite content is observed in the Lp + DMH (4.1 ± 0.6 µm units/mg of protein) group compared to the CAP + DMH (4.9 ± 0.6 µm units/mg of protein) group (Fig. 8.6.C). While, the significant (p<0.05) increase in liver nitrite level is observed in the case of Lp + DMH (2.4 ± 0.3 µm units/mg of protein) group as compared to the CAP + DMH (3.8 ± 0.4 µm units/mg of protein) group (Fig. 8.6.D). It concludes that DMH elevates the catalase and nitrite levels in the colonic region, resulting in tissue damage. On the contrary, Lp prevents the colonic region from oxidative stress damage which may cause by DMH.

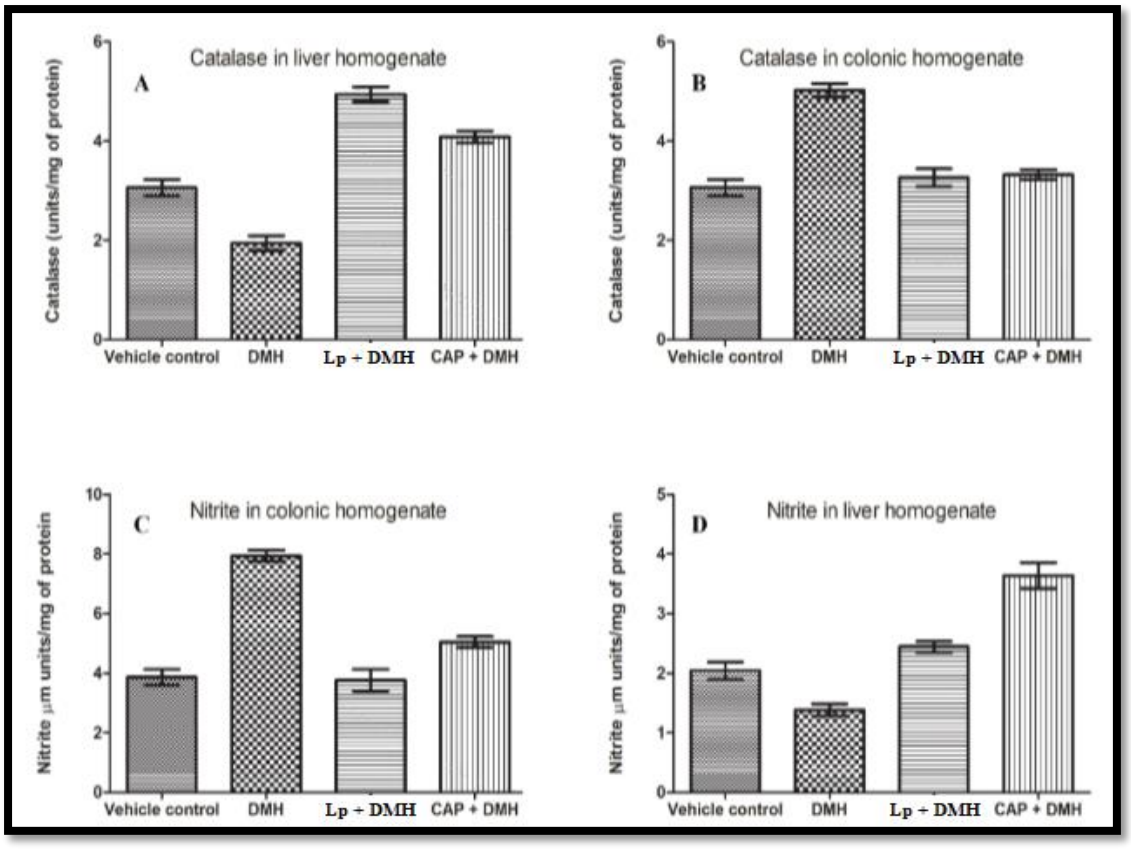

Fig 8.6. Enzyme activities of A) catalase, and D) nitrite in liver homogenate of all group, B) catalase, C) nitrite in the colonic homogenate of all group; data are represented as mean ± SEM of 5 values

DMH is found to have an affinity to colonic tissue resulting in alternation and induction of severe oxidative stress [29]. A significant increase (p<0.05) in liver catalase level in Lp + DMH group as compared to capecitabine-treated group shows strong endogenous antioxidant activity induced by Lp. It is reported that a decrease in catalase activity due to diabetes increase the incidence of DMH induced colonic cancer [30]. Significant increase in the level of colonic nitrite as compared to liver nitrite in DMH treated group suggested that DMH is toxic, specific to a colonic region and not to the liver. The Lp + DMH group shows a significant reduction in colonic and liver nitrite content as compared to the capecitabine-treated group indicating strong antioxidant properties of Lp preventing free radical DNA damage of host. Previous studies showed that an increase in the nitrite content in tissue increases the risk of colonic cancer by altering the cell cycle pathway, observed during the meat preservative studies [31]. It concludes that Lp prevents DMH induced oxidative changes in colonic tissues and acts as a strong antioxidant agent as compared to capecitabine.

Side effects observed in capecitabine treated rats include less progression in weight gain, a severe increase in WBC count and a decrease in lymphocyte count as compared to Lp + DMH group. These side effects are nullified in the Lp + DMH group and thus, *Lactobacillus plantarum* acts as a good antioxidant mediator which is significantly better than capecitabine.

8.3.5 Histopathological evaluation of Lp formulation

The vehicle control group do not show any dysplasia or adenoma conditions (Fig. 8.7.A). The DMH group shows polyps like condition along with ACF formations (Fig. 8.7.B). Mild hyperplasia is observed in the case of CAP + DMH group (Fig. 8.7.D) as compared to the Lp + DMH treated group (Fig. 8.7.C). This condition of hyperplasia is regarded as the initiation of ACF and adenocarcinoma proliferation. Further, the significant (p<0.05) reduction in the count of ACF is noticed in the Lp + DMH group compared to the CAP + DMH group.

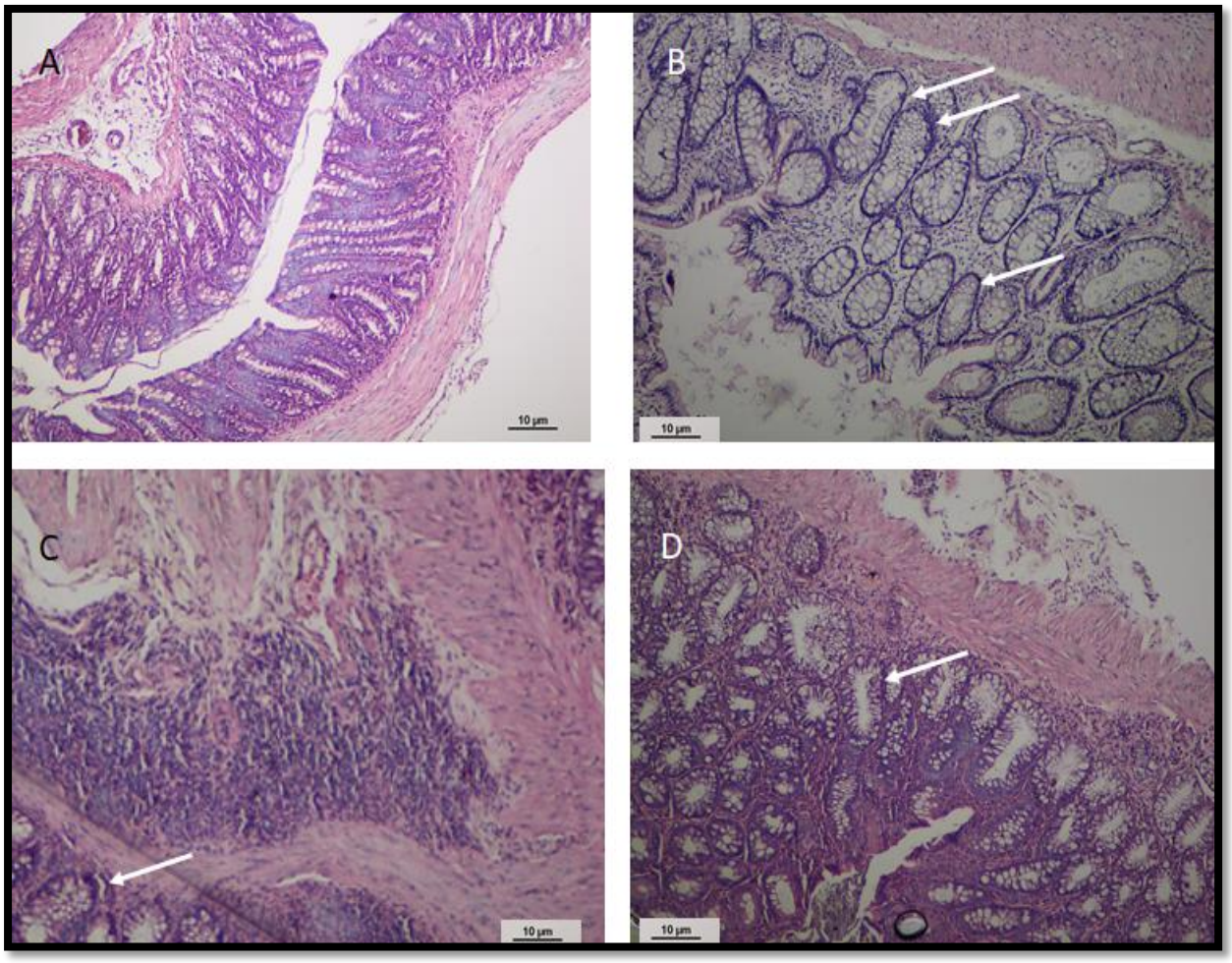

Fig 8.7. Histopathological evaluation of colonic mucosa by hematoxylin & eosin staining. A) vehicle control with no sign of adenomas, B) DMH group with severe dysplasia, adenomas shown by white lines initiation of ACF C) Lp + DMH group with less dysplasia shown with white lines, D) CAP + DMH with less dysplasia but are with crypts.

Thus, it is concluded that Lp protects the colonic cells from DMH, preventing the cells from degradation conditions like severe dysplasia and crypts formations. As, these crypts and ACF formations generally acts as a precursor for adenoma and development of colonic cancers.

8.4 Conclusions

In the present investigations, *Lactobacillus plantarum* isolated from sheep milk is found to be non-toxic nutraceutical agent demonstrated successfully by acute

oral toxicity study. This LAB is found effective in preventing colonic cells from the chemical induced oxidative stress conditions due to DMH, which may led to cancer. This LAB also prevented the alternation in liver and spleen index from oxidative stress effects of DMH. Further, this microbe successfully maintained the functioning of catalase, nitrite enzyme systems along with the increase in the Hb and decrease of WBC level to a threshold as compared to synthetic drug capecitabine. *Lactobacillus plantarum* protected the colonic cells from ACF and adenoma formations due to DMH, revealed by histopathological investigations. Thus, this study concludes that *Lactobacillus plantarum* not only reduced the precancerous colonic lesions but also diminished the chemically induced oxidative stress not seen by treatment of synthetic drugs like the capecitabine and may act as a better functional food against chemically induced colonic cancer.

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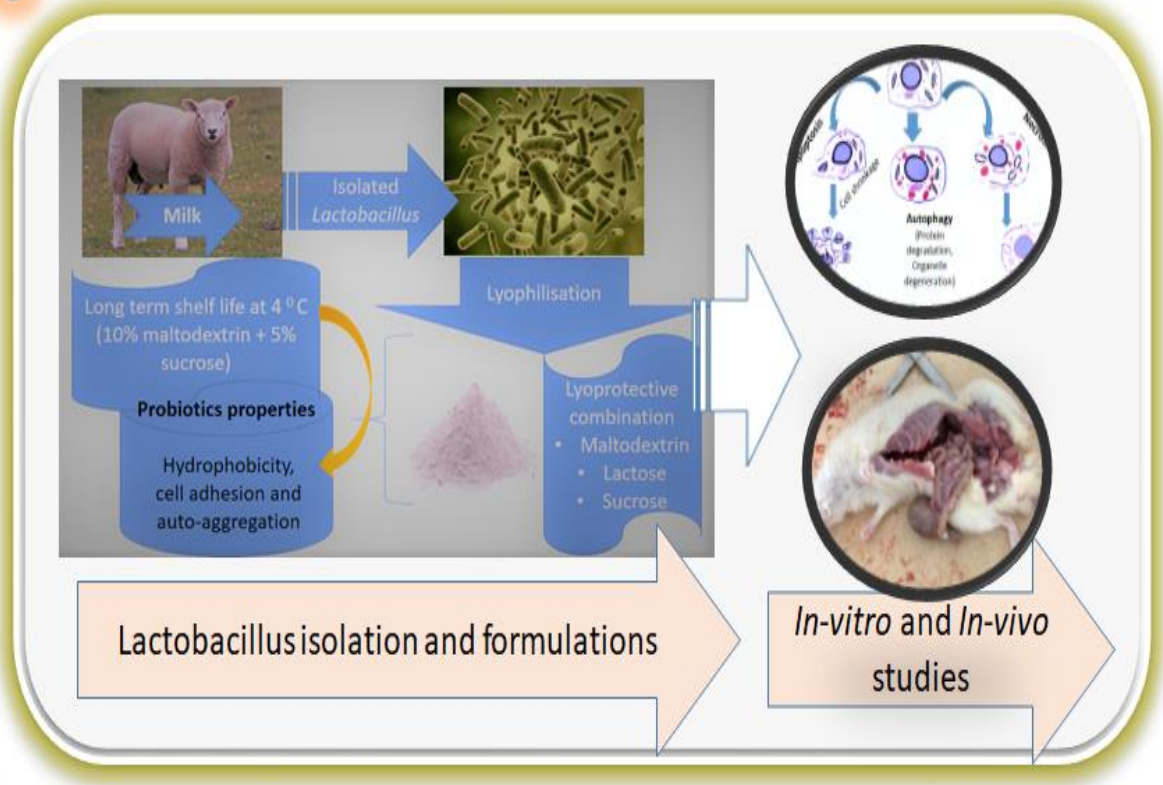
Chapter 9

Eat to beat the Cancer- J. Robert Hatherill

Summary and Conclusions



9.1. Introduction

Probiotics are the basic source of the functional food used as 'yogurt, dahi' but found with the short shelf life. Functional foods are used as the alternative source of healing component by the physiological pathways. These are used as the therapeutical agent along with the diet to reduce the risks of various diseases as a prophylactic agent. The most common diseases that are treated by neutraceutical agents are gastrointestinal diseases and buccal cavity disorders. The pharmacological activities that are observed in case of probiotics are mostly by antioxidant mode of actions without the liver damage and carcinogenesis. The antioxidant mechanisms of probiotics could be due to metal ion chelation, enzyme inhibition and free radical scavenging mechanism. The synthetic drugs that are of the chemical origin or synthesized in the lab precipitate toxicity after long use.

The probiotics especially Lactobacillus genera are extensively explored for a long time as part of the diet. The origin of these microbes is mostly from the milk and its derived products. Since birth, these microbes work together as part of the digestive system as mutualism mode of adaption in the gastrointestinal tract. The probiotics fight the dysbiosis conditions with the pathogenic strains in the lumen of the intestine. In extreme condition, the inoculation of the Lactobacillus is found effective in suppressing and destroying the disease-causing microbes i.e. pathogens. The probiotics include many different bacterial strains, each of these species differs in nature and activity. Most of the times strains of the same species are unique in characteristics such as adherence, immunological activity and other biological actions on the host. The major mechanism involved is the lowering of the pH in the colonic region, inhibiting the adhesion of the pathogenic strains to the intestinal lumen. Auto-aggregation and co-aggregation are the other two mechanisms that are inhibiting the colonization of pathogens. The hydrophobicity of the host-probiotics interaction also prevents various diarrheal diseases. Currently, probiotic research aims in the isolation of new strains and investigation of microbiota and microbiome characteristics in each part of the gastrointestinal tract from the small intestine to the colonic region. The basic focus of research includes the host-microbe interactions

within the intestine, microbe-pathogen interactions within the microbiota and the microbe-drug interactions in diseased condition. The discovery of the new strains of Lactobacillus from the new natural source from the heavy crowd of the microbiota is a tricky and time-consuming process. The mechanism of action shown by the lactobacillus genera and it's sub-strains vary in its pharmacological activity. By the genotypic phylogeny study, all of Lactobacillus strains may fall under the same ancestral category but varies in the biochemical and physiological activities. This indicates that newly discovered strain of Lactobacillus may come with new pharmacological activity against different diseases. The whole genomic sequencing of newly discovered culture highlights the potential of the strain as a functional food in treating many dreadful disorders of the intestine. The positive interaction seen in all the above cases is mediated by means of the antagonistic activity of these probiotics.

Currently, in the market, there is a need for such nutraceutical products which may provide health benefits. Mostly the world is suffering from dreadful diseases like cancer. There are various types of cancer cases, in which colon cancer ranks third in India. The prophylaxis measures available for cancerous diseases are nearly zero as the etiology of the disease is unknown. However, the probiotics are proven useful in treating the diseases of the gastrointestinal tract such as diarrhoea and minor infection or manifestation by the pathogenic strains. Improper bowel habit is found responsible for causing the disease called adenomatous carcinoma a hybrid type of colorectal cancer. This gives the major clue that the Lactobacillus may be used as the major source to heal any kind of the disease including colonic cancer. The drawbacks of using the Lactobacillus as functional food is its short shelf-life. Further, it gets contaminated if kept in a liquid formulations mode. So the marketing of this product is not only a risk factor to the manufacturer but also costly due to its preservation parameters such as refrigeration. Few of the products are nowadays available as dry powder format. They are coming with use of the coat of synthetic materials which is increasing the cost of the product. The use of natural drying material is the solution to overcome this cost in production but are rigid and require

multiple time optimisation of the formulation. If the product gets developed the shelf life is the second issue or hurdle in large scale manufacturing to satisfy the needs of such a large population of our country.

Based on the above-said problems, hypothesis and literature survey the current research is carried out and the outcomes of our studies is reported in the form of nine chapters as- Chapter 1- Introduction, Chapter 2- Milk as "Pre and probiotics": A theoretical background, Chapter 3- Isolation and identification of Lactobacillus from sheep milk, Chapter 4- *In-vitro* gastrointestinal simulated studies of Lp, Lr and La as Lactobacillus, Chapter 5- Studies of the flourishing of Lactobacillus in different types of milk and their Lp, Lr and La formulations, Chapter 6- *In-vitro* antioxidant and supportive antagonistic activities of Lactobacillus, Chapter 7- *In-vitro* colon cancer cell line cytotoxicity and anti-colon cancer activity of Lp, Lr, and La, Chapter 8- *In-vivo* antioxidant and anti-colon cancer activity of Lactobacillus (Lp), Chapter 9- Summary and conclusion.

9.2. Competent bits of the research outcomes

The main aim of the present work was to find a new culture of LAB with probiotics nature showing the antioxidant and anti-colon cancer properties. Earlier, the existing deposited cultures like *L. acidophilus* are found with the nearly zero pharmacology activities. This shows that existing cultures of LAB lacks the potential of active pharmacological activity and also there is a need for discovery of new strains and sub-strains in this category. The main source of LAB is the milk of different milking animals. Thus, the new sub-strains of LAB are isolated from sheep milk. The physiological and biochemical investigations were the clue in the identification of the Lactobacillus at the lab level. Four strains of LAB are isolated by conventional lab methods from which two strains i.e. *L. rhamnosus* and *L. plantarum* are discovered successfully. The identifications of the LAB are done by 16 S rDNA. To explore the differences between the two isolated strains and the existing Lactobacillus strain obtained from NCIM; whole genomic sequencing are carried out. The preservation of both strains was carried out in the NCMR, Pune

which was registered by the name of the D. Y. Patil University Kolhapur, India. Both the isolated cultures are found tolerant of the intestine stress condition like pH, bile salt, NaCl concentration which are analysed by *in-vitro* gastrointestinal simulation studies. Further, these isolates are found with additional properties of cholesterol assimilation, lysozyme survival, haemolytic and DNAse activity. This shows that both the isolates are with the potential of sustaining the physiological stress condition of the human gut.

After isolation, the most important part is the flourishing of LAB in natural growth media. Mostly the existing marketed probiotics are grown in artificial growth media which is not only costly but may precipitate toxicity after prolonged use in the host. On the contrary, both isolates are showing active growth and flourishing in milk as natural media. Our results show that the growth of all LAB is optimum in the milk of buffalo as compared to the milk of sheep, cow, and goat. The milk of various breeds of buffaloes is screened further for viability and growth parameters of LAB. The growth of LAB is found optimum in Mehsana milk as compared to the Bhadwari and Nagpuri milking water buffaloes.

For proper utilisation of the LAB as an end product, it is needed to be converted into a dosage form with good viability and shelf life. The shelf life of LAB can be enhanced by granule or powder formulations. In addition, very few probiotics formulations are available in the market which comes in granule or powder formulations by use of pharmaceutical excipients. Currently, the marketed probiotics formulations come in liquid dosage forms with short shelf life and involve refrigerations. The use of pharmaceutical excipients to enhance the shelf life of LAB by granule formulations is a tricky process which involves proper optimizations for ease of manufacturing and end-user satisfactions, else it causes heavy product losses. The cost of the products may increase if the final products as formulations are not properly optimised. In the case of our product, both spray dry and lyophilization techniques are employed. The study shows that the use of maltodextrin in combinations with starch or lactose enhances shelf life. Comparative studies are carried out in the case of two LAB strains for long-term stability testing. The

lyophilized products are screened for a period of 12 months at two different extreme conditions i.e. at 4 ^{o}C and at 37 ^{o}C. In the cases of all strains, significantly higher survival (p<0.05) rate are found at 4 ^{o}C as compared to 37 ^{o}C. Further, hygroscopicity is found low in case of *L. plantarum* with the formulation F4 and increases significantly in *L. acidophilus* formulation F12 observed by a spray dried encapsulation technique. Similarly, spray dried products are screened for a period of 12 months at two different extreme conditions i.e. at 4 ^{o}C and at 37 ^{o}C. Combinations of maltodextrin and starch in ratio 2:1 shows greater viability as compared to other pharmaceutical excipients in combinations. A decline in shelf life or no viabilities are observed in cases of all LAB after 6 months when formulations are kept at room temperature at 37 ^{o}C. All the summarised results show that LAB as dry powders and granules acts as an ideal formulations with the long shelf life.

These encapsulated formulations keep the LAB viable, protecting the probiotics antioxidant and its antagonistic activities. Further, our LAB is screened for antioxidant and supportive antagonistic activities by various assays. The antioxidant activities determined by the scavenging of hydroxyl radicals, DPPH and other assays show that the *L. plantarum* as lysate with significantly greater scavenging activity than an intact cell as compared to both *L. rhamnosus* and *L. acidophilus*. The zone of inhibition studies was carried out for LAB against all tested Gram positive and Gram-negative microorganism. The maximum zone of inhibition is observed for *L. plantarum* as the whole organism as compared to lysate and supernatant. This revealed that the antagonistic activity of *L. plantarum* is highest as compared to the reference standard *L. acidophilus*. Similarly, adherence abilities of LAB isolates of different formulations of spray dry (F1 - F12) and lyophilization (F13 - F30) granules were tested in the Red snapper fish intestinal mucus. The results show that *L. plantarum* adhered significantly higher than *L. rhamnosus* and *L. acidophilus*. Auto-aggregation studies are carried out in LAB formulations in which *L. plantarum* F19 formulation after lyophilisation shows maximum auto-aggregation than formulation F13 of *L. rhamnosus*. In conclusion, *L. plantarum* shows higher auto-aggregation and adhesion properties as compared to rest other

Lactobacillus strains. This property of auto-aggregation is additional antagonistic pathway assisting the antioxidant activity observed strongly in *L. plantarum* JDARSH strain.

Cell line studies are carried out using LAB, as the drug or nutraceutical products. The formulations or drug decreasing the viability of cancer cell lines is considered as an ideal candidature to heal the cancer disease. The viability study conducted by SRB and MTT assay on HCT 115 cell line show a decrease in percentage cell viability in case of the *L. plantarum* for 50 µg/ml dose as compared to other LAB under trails. Thus, it revealed that *L. plantarum* as an effective antiproliferative agent against HCT 115 cell line after 48 h. The significant apoptotic signals are observed in the case of *L. plantarum* as compared to *L. rhamnosus* and *L. acidophilus* observed by DAPI assay. Similarly, in the clonogenic assay, *L.* plantarum shows a cytotoxic effect on the colon cancer cell line HCT 115 as compared with other Lactobacillus reference strains. FITC- conjugated annexin V proves the mechanism of action of *L. plantarum* with the apoptosis at late apoptosis phase as compared to rest other strains of Lactobacillus. The apoptotic proteins are investigated by the up-regulation and down-regulation of the Bax, Bcl2 and beta-actin along with its expression ratio. *L. plantarum* shows the apoptotic activity by up-regulation of Bax and down-regulation of Bcl2 gene. Based on these results *L. plantarum* was selected further for animal studies.

Chemical-induced oxidative stress studies are carried out using *L. plantarum* on Wistar rat's anti-colon cancer model. Very few natural origin drugs are available in the market that resists the chemical induced oxidative stress conditions. These cellular stress conditions if not controlled, precipitate cancer in the host. The present study apportioned the *L. plantarum* as a natural antioxidant mediator, as it prevented the DMH elevated chemical-induced oxidative stress condition observed in Wistar rats. The cfu count of probiotics above $10^{7\text{-}8}$ is found as a therapeutic concentration responsible for showing the pharmacological activities. In the present study, the food intake of animals treated with *L. plantarum* group are found higher as compared to the synthetic drug capecitabine group, as a reduction in food intake is

observed in case of DMH treated animals. This reflects that DMH does not affect the physiological metabolism of *L. plantarum* treated group and the values found are the same as that of the vehicle control group. Further, it shows that *L. plantarum* not only maintained the proper bowel condition of the host but also protected it from oxidative stress-inducing chemicals like DMH. The *L. plantarum* group shows a significant increase in body weights after 6 weeks period which are the same as the vehicle control group, not observed in case of the synthetic drug group. This indicates that the weight gain using *L. plantarum* is higher than using the synthetic drug during the whole study protocol. The sluggish weight gain in the synthetic drug group is due to loss of appetite in animals and can be considered as a side effect of this synthetic drug. The hematological evidence shows that the Hb level is decreased with an increase in WBC count by DMH, which may further lead to cancer or even other dreadful diseases. The decrease in Hb level results in anemia and is found to be greatly affecting the pathophysiological conditions of a cancer patient. The present study shows that *L. plantarum* not only increased the Hb level but maintained neutrophils and lymphocyte count nearly the same as a vehicle control group. The administration of DMH is found associated with the increase in the WBC count and altering the microenvironment of the tissue. The significant increase in the RBC value of *L. plantarum* group is observed higher as compared to the synthetic drug group. The ACF is identified by methylene blue staining of the colonic region which acts as the precursor of adenoma and is observed in case of DMH treated group. ACF count observed in the case of the *L. plantarum* group is significantly lower as compared to the synthetic drug group observed by the histopathological investigation. This shows that *L. plantarum* scavenged the oxidative stress from the cell, preventing ACF formations and is more DMH induced oxidative stress protectant than capecitabine. In liver and spleen indices studies DMH drastically affected the colonic regions with minor effects on liver cells. This indicates that *L. plantarum* prevents the splenomegaly conditions. No any sign of hepatomegaly is observed in *L. plantarum* treated group indicating normal liver functioning.

DMH is found to have an affinity to colonic tissue resulting in alternation and induction of severe oxidative stress. A significant increase in liver catalase level in *L. plantarum* group as compared to the synthetic drug-treated group shows strong endogenous antioxidant activity induced by *L. plantarum*. It concludes that *L. plantarum* prevents DMH induced oxidative changes in colonic tissues and acts as a strong antioxidant agent as compared to the synthetic drug group. Side effects observed in synthetically drug-treated rats include less progression in weight gain, a severe increase in WBC count and decreases in lymphocyte count as compared to *L. plantarum* group. Thus, these side effects are not observed in *L. plantarum* treated group which acts as a good antioxidant mediator significantly better than capecitabine.

Thus, *in-vivo* studies revealed that *L. plantarum* not only reduces the precancerous colonic lesions but also diminishes the chemically induced oxidative stress conditions, which is not observed by treatment with the synthetic drug. Thus, *L. plantarum* formulations may be considered as ideal neutraceutical product to heal adenomatous chemical induced carcinoma at the lowest cost and may act as a natural origin functional food.

9.3. Major conclusions

The unistrain probiotics i.e. *L. plantarum* are developed successfully as a dry granule formulations. The culture of *L. plantarum* showed both anti-colon cancer and antioxidant activities. The basic studies and satisfactory results obtained from the dry granules of *L. plantarum* and *L. rhamnosus* in the present thesis work leads to the following conclusions:

1) Initially, the isolation of *L. plantarum* and *L. rhamnosus* are successfully carried out from the sheep milk with deposition of the culture in the public domain, the government of India.
2) The successful DNA isolation of the *L. plantarum* and *L. rhamnosus* by gel electrophoresis are demonstrated with whole genomic sequencing of the culture.

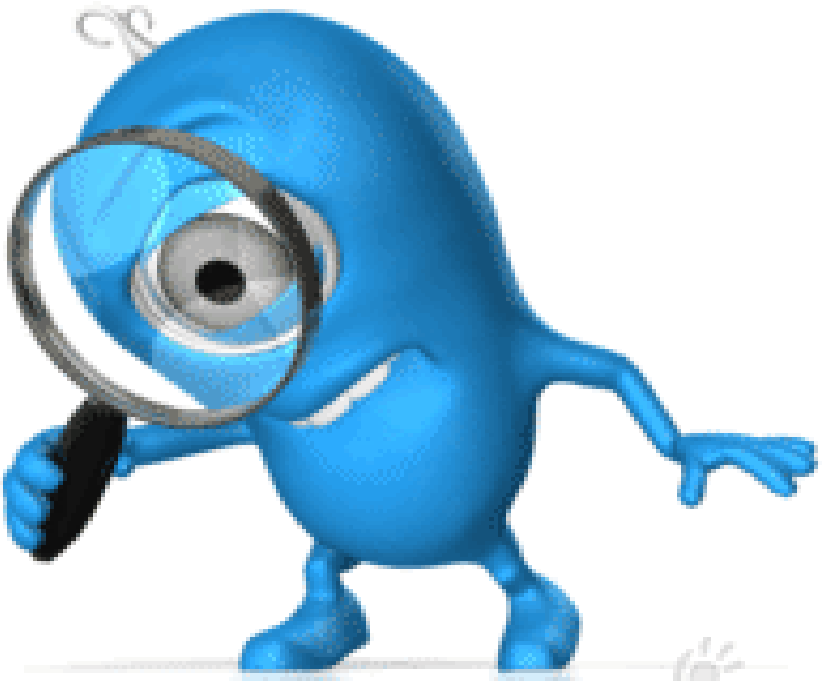
3) The isolated strain specifically *L. plantarum* fulfilled all the conditions to be good probiotics by showing tolerance to simulated stress condition of a gastrointestinal tract such as bile tolerance activity, cholesterol assimilation, resistance to phenol, lysozyme resistance, BSH Activity, response to SSDP, hemolytic activity and DNase activity
4) The isolated both strains showed maximum growth rate in buffalo's milk as compared to other milking animal's milk. The maximum growth rate are observed in the milk of Mehsana water buffalo's.
5) The formulations of optimised dry granules are developed by lyophilisation and spray dry methods successfully using basic pharmaceutical excipients.
6) *L. plantarum* showed higher antioxidant and supporting antagonistic probiotics properties as compared to other isolated strain.
7) *In-vitro* anticancer studies showed that *L. plantarum* with greater cancer cell extinction activity demonstrated by DAPI analysis, MTT and SRB assay, clone formations against HCT 115 cell line, Annexin V/PI analysis and by up/down-regulation of the apoptotic protein expressions, as compared to *L. rhamnosus*. Based upon these results, *L. plantarum* is considered a strong candidate for *in-vivo* animal studies.
8) *In-vivo* studies revealed that the *L. plantarum* as nutraceutical antioxidant and anti-colon cancer mediator proven by enzyme analysis along with haematological investigation supported by the liver marker, fecal matter analysis, and histopathological studies.

In conclusion, the unistrain *L. plantarum JDARSH MCC 3959* is developed for first time in granule form with the anti-colon cancer and antioxidant activities. These granules are having the advantage over the liquid formulations in aspects of shelf life, durability and cost effectiveness.

9.4. Future prospective

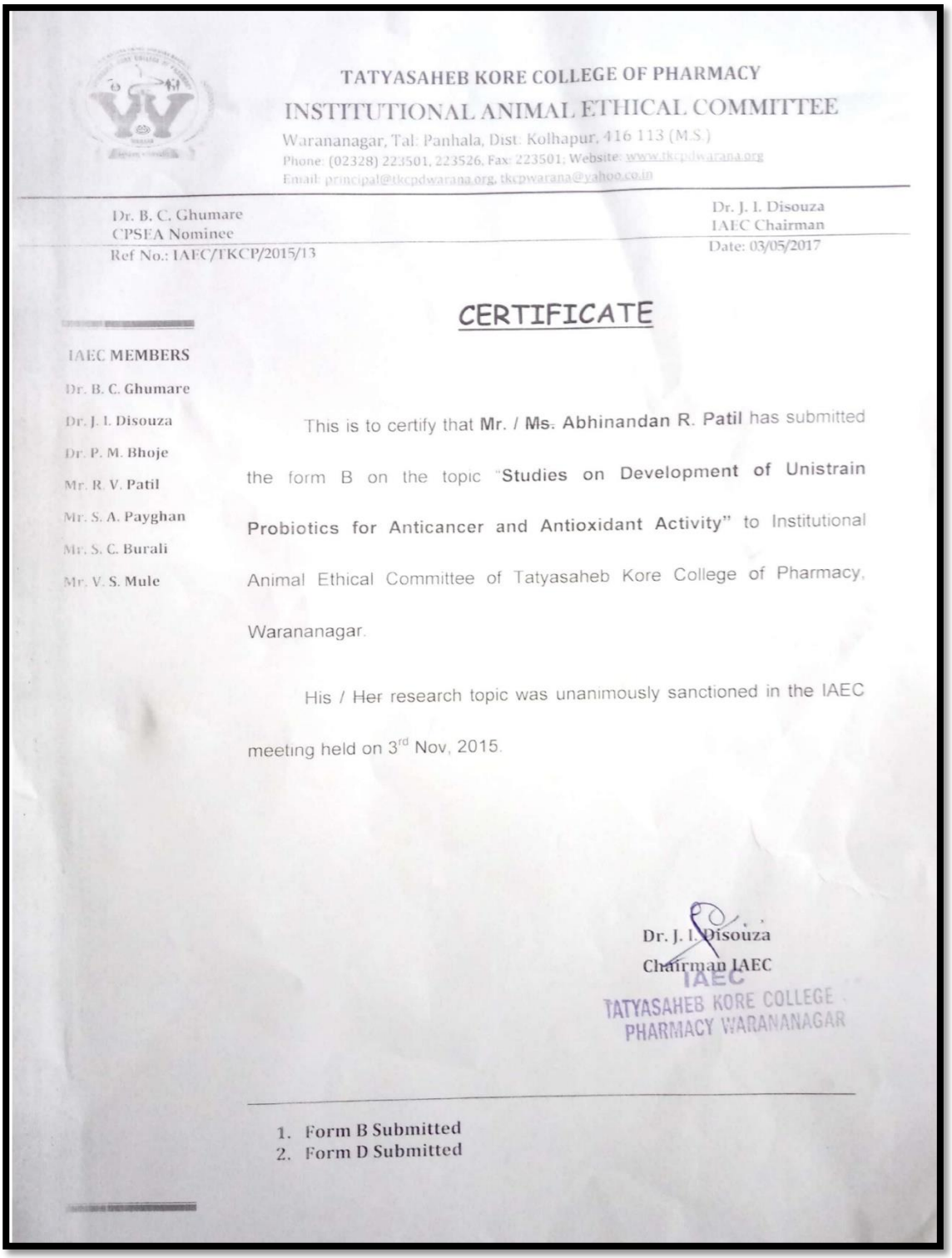
In continuation with the work carried out for Ph.D. thesis, the product developed in the form of granules is having many more advantage over the conventional products. This will be only first kind of neutraceutical product that will

be cost effective, easy to formulate and come with longer shelf life and can be made available as small sachets in mall, shops, and canteen. This will be further studied by carrying out the basic clinical trials as per norms of nutraceutical regulation and AYUSH government of India, the product can be brought into the market.



APPENDIX

APPENDIX-A: IAEC CERTIFICATE



TATYASAHEB KORE COLLEGE OF PHARMACY
INSTITUTIONAL ANIMAL ETHICAL COMMITTEE
Warananagar, Tal: Panhala, Dist: Kolhapur, 416 113 (M.S.)
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Dr. B. C. Ghumare
CPSEA Nominee
Ref No.: IAEC/TKCP/2015/13

Dr. J. I. Disouza
IAEC Chairman
Date: 03/05/2017

IAEC MEMBERS
Dr. B. C. Ghumare
Dr. J. I. Disouza
Dr. P. M. Bhoje
Mr. R. V. Patil
Mr. S. A. Payghan
Mr. S. C. Burali
Mr. V. S. Mule

CERTIFICATE

This is to certify that Mr. / Ms. Abhinandan R. Patil has submitted the form B on the topic "Studies on Development of Unistrain Probiotics for Anticancer and Antioxidant Activity" to Institutional Animal Ethical Committee of Tatyasaheb Kore College of Pharmacy, Warananagar.

His / Her research topic was unanimously sanctioned in the IAEC meeting held on 3rd Nov, 2015.

Dr. J. I. Disouza
Chairman IAEC

IAEC
TATYASAHEB KORE COLLEGE
PHARMACY WARANANAGAR

1. Form B Submitted
2. Form D Submitted

APPENDIX-B: NCMR CERTIFICATE FOR CULTURE DEPOSITION

NCMR
राष्ट्रीय सूक्ष्मजीव संपदा केंद्र

National Centre for Microbial Resource
Member of WFCC, An IDA under Budapest Treaty, DNR of MoEF
राष्ट्रीय कोशिका विज्ञान केंद्र | National Centre for Cell Science

❖ CERTIFICATE OF DEPOSIT ❖

***Lactobacillus plantarum*. Strain JDARSH**

was received for public access deposit at NCMR from

Mr. Abhinandan R. Patil, Dr. John Disouza, Dr. Shivaji H. Pawar,

D.Y. Patil University, Centre for Interdisciplinary Research, Kolhapur- 416006,
Tatyasaheb Kore College of Pharmacy, Warananagar, Tal- Panhala, Dist. Klohapur- 416113

On **18 January 2018**

and was allocated the accession number

MCC 3595

After confirming the viability, purity and authenticity of the strain in the NCMR facilities, it has been preserved using standard methods.

The strain is now available to the public.

Dr. Amaraja Joshi

Scientist, Microbial Culture Collection, Pune

12 March 2018

साई ट्रिनिटी कॉम्प्लेक्स, सुस रोड, पाषाण, पुणे–४११ ०२१. महाराष्ट्र, भारत.
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एनसीसीएस भारत सरकार के जैवप्रौद्योगिकी विभाग का स्वायत्त संस्थान है.
NCCS is an Autonomous Institute of Department of Biotechnology, Govt. of India

NCMR
राष्ट्रीय सूक्ष्मजीव संपदा केंद्र

National Centre for Microbial Resource
Member of WFCC, An IDA under Budapest Treaty, DNR of MoEF
राष्ट्रीय कोशिका विज्ञान केंद्र | National Centre for Cell Science

❖ CERTIFICATE OF DEPOSIT ❖

***Lactobacillus rhamnosus* Strain ARJD**

was received for public access deposit at NCMR from

Mr. Abhinandan R. Patil, Dr. John Disouza, Dr. Shivaji H. Pawar,

D.Y. Patil University, Centre for Interdisciplinary Research, Kolhapur- 416006,
Tatyasaheb Kore College of Pharmacy, Warananagar, Tal- Panhala, Dist. Klohapur- 416113

On **18 January 2018**

and was allocated the accession number

MCC 3594

After confirming the viability, purity and authenticity of the strain in the NCMR facilities, it has been preserved using standard methods.

The strain is now available to the public.

Dr. Amaraja Joshi

Scientist, Microbial Culture Collection, Pune

12 March 2018

साई ट्रिनिटी कॉम्प्लेक्स, सुस रोड, पाषाण, पुणे-४११ ०२१. महाराष्ट्र, भारत.
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NCCS is an Autonomous Institute of Department of Biotechnology, Govt. of India

At Curriculum Vitae

- Name : Abhinandan R Patil
- Date of Birth : 29 Nov 1988
- Gender : Male
- Marital Status: Married
- Languages : English, Hindi, Marathi, Kannada
- Nationality : Indian
- Residential Address: 115A/E Sykes Extention Near Law College Kolhapur 416001
- Email Address : abhisir5@gmail.com
- Contact No. :+919511761008

CAREER OBJECTIVE:-

A stride of excellence at beginning of career in pharmaceutical global practice with developed multifaceted skills. Brusquely fulfil the requirement of organization and get the key position in organization.

EDUCATIONAL DETAILS

Course	Institute	Month & Year of passing	Percentage
Ph.D.	**D. Y. Patil University, Kolhapur DST INSPIRE SRF/2017.**	**2019**	**(Thesis submitted)**
M-PHARM	**Shivaji University, Kolhapur**	Sept 2013	**98.00 (Gold medalist)**
GPAT (Pharmacy)	**Indian Institute of Science, Bangalore**	Feb 2011	75.2 MARKS.
B. Pharm	**Shivaji University, Kolhapur**	April 2011	75.40 **(Gold medalist)**

INNOVATIVE INVENTIONS:

a) Discovered new strain of Lactobacillus strain and culture deposited at National Centre for Microbial Resource as ***Lactobacillus rhamnosus*** **ARJD, MCC 3594.**

b) Discovered new strain of Lactobacillus strain and culture deposited at National Centre for Microbial Resource as ***Lactobacillus plantarum*** **JDARSH, MCC 3595.**

c) Registered genomic bank accession number of *Lactobacillus plantarum* JDARSH is PYBS01000000.

d) Registered genomic bank accession number of *Lactobacillus rhamnosus* ARJD is PYBS00000000.

RESEARCH PUBLICATIONS:

7 International publication, 1 book chapters, 1 review

1) **Patil A. P**, Disouza J. D, Pawar S. H. (2019) Shelf life stability of encapsulated lactic acid bacteria isolated from Sheep milk thrived in different milk as natural media. Small Ruminant, 170, 19-25.

2) **Patil A. P**, Disouza J. D, Pawar S. H. (2019) Granules of unistrain Lactobacillus as nutraceutical antioxidant agent. International Journal of Pharmaceutical Sciences and Research. 9 (4), 1594-1599.

3) **Patil A. P**, Disouza J. D, Pawar S. H (2018) Health benefits of probiotics by antioxidant activity: A review. Pharma Times 50 (9), 1-3.

4) **Patil A. P**, Disouza J. D, Pawar S. H (2018) Probiotic potential of *Lactobacillus plantarum* with the cell adhesion properties. Journal of Global Pharma Technology 10 (12), 1-6

5) **Patil A. P**, Disouza J. D, Pawar S. H (2018) *Lactobacillus acidophilus* as a Preferable Natural Anticancer Agent. SM Journal of Pharmacology and Therapeutics 3 (1), 1019

6) **Patil A. P**, Disouza J. D, Pawar S. H (2018) Probiotics with Dimethyl Hydrazine Induced Animal Study as New Psychological Study Model, International Journal of Animal Science, 2 (2): 1017.

7) Tande A, **Patil A. P**, et. al., (2016) Lactobacillus Model Moiety a New Era Dosage Form as Nutraceuticals and Therapeutic Mediator, in: Biotechnology and Bioforensics, Springer, Singapore11–21.

8) Tande A, **Patil A. P,** et. al., (2016) Screening of soy lectin: as new era cancer healing agent International Journal of Pharmaceutical Sciences and Research 7 (5), 2147

9) Rustomjee M, **Patil A. P**, et. al., (2016) Pharmaceutical Product Development: Insights into Pharmaceutical Processes, Management and Regulatory Affairs, CRC Press, 393-405.

10) **Patil A. P**, Disouza J. D, Pawar S. H (2019) Probiotic potential of *Lactobacillus plantarum* with the antioxidant properties, International Journal of Pharmacy and Biological Sciences (Submitted).

11) **Patil A. P**, Disouza J. D, Pawar S. H (2019) Complete genome sequencing of *Lactobacillus plantarum* JDARSH isolated from sheep milk, Probiotics and Antimicrobial proteins (Submitted).

AWARDS

A) INTERNATIONAL

1) **Best paper award** at International Conference on Infectious disease at 5th Indo-Japanese symposium, **Tokyo Japan**. 2014.

2) **Young Scientist Award,** at International Conference on "Bio-informatics, Biology, Forensic science" Organized by **Bio-axis, Hyderabad** 2013.

B) NATIONAL

1) Awarded with Gold medal by IPA (Indian pharmaceutical association) for 1st rank in Shivaji University 2011.
2) Awarded with **J.P.MODI Best excellence student** award from IDMA (INDIAN DRUG MANUFACTUERE ASSOCIATION) 2012.
3) Recipient of **Best outstanding student award** (BOS) from all India: IPC TRUST (Indian Pharmaceutical Council Trust) 2012.
4) Winner of the Industry-University Summit as a part of Avishkar from all age group 16-70**,** Feb 2013: Aurangabad.
5) Secured first position at **National level poster presentation** event at Kasegoan, for "Schedule Hx: need to upgrade Indian pharmacist" 2012.
6) Winner at National Level model presentation at Kasegaon ICMR (Indian Council of Medical Research) "Dermoelectron". March 2013.
7) Winner of Abhinav Foundation Research competition conference at Mumbai, from all age groups from 24 to 60 years, 2014.
8) Recipient of **first prize** in Poster presentation at Anveshan 2015-16 at west zonal level Bikaner, Rajasthan for "Epitope analyser for colorectal cancer". Jan 2016.
9) Recipient of **first prize** in Poster presentation at Anveshan 2015-16 at Central level Anand, Gujrat for "Epitope analyser for colorectal cancer". March 2016.
10) Recipient of **first prize** in Oral presentation at National conference "Nanoscience" Warananagar 2018.
11) Secured first position at **National level Oral presentation** event at Warna for research project on "Epitope analyser for Colon Cancer" 2012.
12) Secured **first** position at **National level poster presentation** event at Peth-vadgoan, for "MEDI-VED: Master card for pharmacist appraisal" 2012.

13) Recipient of **first prize** at National Conference on Emerging Technology (NCET) Shivaji University Kolhapur, Jan 2013.
14) Recipient of **first prize** at National Conference on Applied microbiology for Globe Protection on topic "Probiotics and eco-system" Y. C Satara 2013.
15) Recipient of **first runner up** prize on topic "Spray dried milk chocolate as New antacids", UGC Sponsored Multidisciplinary approaches in Microbiology, at Rajaram College, Kolhapur, 2014.
16) Recipient of **first runner up** prize at **National level FDDPR** event at BHARATI Vidyapeeth College of pharmacy Kolhapur. 2010.
17) Secured **second** position at National level Elocution Competition, Organized by Indian Pharmaceutical Association, at School of Pharmacy, SVKM'S, NMIMS, Mumbai 2012.
18) Secured **second** position at National level poster presentation event at Peth-vadgoan, for "Model presentation on Anti-Aids prevention research" 2012.
19) Won **second prize** at FDDPR (National level) conference at Bharati Vidyapeeth college of Pharmacy "Nanoviricides" 2012-13.
20) Recipient of **second runner up prize** on "Analytic technique development" at Pune Bharati Vidyapeeth College of pharmacy, Pune 2013.

C) STATE LEVEL

1) Recipient of **Startup HERO OF MAHARASHTRA STATE "STARTUP INDIA" Under Make in India** scheme 2018.
2) Recipient of award "**Cancer awareness personality of Kolhapur**" from Mahavir Education Society, Kolhapur 2018.
3) Recipient of **Ideal model** presenter award at Govindrao Nikam College of Pharmacy, Sawarde, 2011.
4) Recipient of **first prize** in oral presentation at Satara as state level competition for Research project of "stem cells" 2012.
5) Recipient of **first prize** at State level Research paper presentation at Yashoda College, SATARA: 2012-13: Feb 2013.
6) Recipient of **first prize** at State level Conference Rajaram College, "On H1N1 Vaccine generation model, Kolhapur 2013.
7) Recipient of **first runner up prize** at state level technical poster presentation event PIONEER 2K6, KITE Kolhapur. 2011.
8) Recipient of **first prize** in **Poster** presentation at **Avishkar** at inters college level for "Reverse vaccinomics: H *pylori.* 2010.

9) Recipient of first prize in **Poster** presentation **Avishkar** at University level for "Reverse vaccinomics- *Vaccine development against H pylori".* 2010.
10) Recipient of **first prize** in **oral** presentation at **Avishkar** at inter-college level for "Reverse vaccinomics: H *pylori"* 2010.
11) Recipient of first prize in Poster presentation **Avishkar** at inter-college level for "New generation Epitope analyser for Breast cancer" 2011.
12) Recipient of **first prize** in Poster presentation **Avishkar** at inter-city level for "New generation Epitope analyser for Breast cancer" 2011.
13) Recipient of **first prize** in Poster presentation **Avishkar** at inters college level for "New Era Epitope Finger print technique for diagnosis and prognosis of Colon cancer" 2012.
14) Recipient of **first prize** in Poster presentation **Avishkar** at intra university level for "New Era Epitope Finger print technique for diagnosis and prognosis of Colon cancer" 2012.
15) Recipient of **first prize** in Poster presentation **Avishkar** at state level for "New Era Epitope Finger print technique for diagnosis and prognosis of Colon cancer" 2012.
16) Recipient of **first prize** in Poster presentation **Avishkar** at inters college level for "Multi-axial Epitope Finger print technique for diagnosis and prognosis of colorectal cancer" 2013.
17) Recipient of **first prize** in Poster presentation **Avishkar** at Intra University level for "Multi-axial Epitope Finger print technique for diagnosis and prognosis of colorectal cancer" 2013.
18) Recipient of **first prize** in Poster presentation **Avishkar** at Inter-College level for "Breast Cancer diagnosis and prognosis kit" 2014.
19) Recipient of **first prize** in Poster presentation **Avishkar** at Intra University level for "Multi Breast Cancer diagnosis and prognosis kit" 2014.
20) Recipient of **first prize** in Poster presentation **Avishkar** at Inter College level for "Colon cancer nanotheronostics" 2015.
21) Recipient of **first prize** in Poster presentation **Anveshan** at Inter College level for "Epitope analyser for colorectal cancer" 2015.
22) Recipient of **first prize** in Poster presentation **Avishkar** at university level Pune for "Colon cancer nanotheronostics" 2016.
23) Recipient of **Best out Standing Student award** at college Tatyasaheb Kore College of pharmacy Warananagar 2013.

24) Awarded as Best Outstanding Student (B.O.S) of Bharati Vidyapeeth College of pharmacy Kolhapur. 2010.
25) Best outstanding student at school **Irwin Christian English medium school** 2004.
26) Received **Mr. TAKCIP,** year 2011-12, Ideal and all-rounder student award from college. 2012.
27) Received **Mr. TAKCIP,** year 2012-13, Ideal and all-rounder student award from college. 2013 **(Hat-trick).**
28) Secured Second position at Debate competition held at Rajaram **College** Kolhapur 2009.
29) Secured **first prize** in quiz competition in "mantra", Kolhapur 2009.

EXTRA- CURRICULAR ACTIVITIES:

1) Actively participated in state level elocution competition in Satara College of Pharmacy, Satara. 2009.
2) Runner up in District level quiz competition 'Maantan kolhapur 2009'.
3) Participate in District level intercollegiate Debate competition in Maantan kolhapur 2009'.
4) Participate in District level intercollegiate Elocution competition in Maantan kolhapur 2009'.
5) Participate in District level intercollegiate Parliament assembly competition in Maantan kolhapur 2009'.
6) Actively participated in state level elocution competition in Satara College of Pharmacy, Satara. 2013.
7) Different prizes in district level in drawing at higher secondary level.
8) Different prizes in district level in elocution at higher secondary level.
9) Actively participated and winner in sports and at undergraduate level.
10) Actively participated and winner in sports and at Post graduate level.

INSTRUMENT KNOWLEDGE:-

U. V. Spectrophotometer – Shimadzu 1800, I. R. Affinity 1 – Shimadzu, Disso 2000 – Lab India, Gene runner software, ELISA kit, Spray dryer JISL Minispray.

SEMINAR, CONFERENCES AND WORKSHOPS ATTENDED:

International

1) Industrial current scenario at Mumbai, by IDMA "Indian" 2012.
2) Conference on food processing at Tanjavur, T.N., 2013.
3) Conference on "Bio-informatics, Biology, Forensic science" Organized by Bio-axis, Hyderabad 2013.
4) Conference on "Infectious disease at 5^{th} Indo-Japanese symposium", Tokyo Japan. 2014.
5) Indo-Japanese International Symposium, Goa, 2016.

6) Conference on Nanotechnology, Material science, D Y Patil education society, 2017.
7) Conference at Kolhapur, on "Sustainable water resource development and management" 2012.
8) International capacity building workshop by National Council of Science Museums, Kolkata, 2015.

National

1) 62th Indian Pharmaceutical Congress, at New Delhi, December 2010.
2) Bio-vision microbiology seminar at Rajaram College 2009.
3) "Memory enhancement" workshop by Murli khernar 2009.
4) **1st National level** conference **of FDDPR** at Bharati Vidyapeeth College of pharmacy, Kolhapur 2009.
5) **2nd National level** conference **of FDDPR** at Bharati Vidyapeeth College of pharmacy, Kolhapur 2010.
6) **5th** Inter-university Conference and Research competition at Nashik, 2010.
7) **3rd National level** conference **of FDDPR** at Bharati Vidyapeeth College of pharmacy, Kolhapur 2011.
8) **6th** Inter-university Conference and Research competition at Kolhapur, 2011.
9) Herbal conference at R.C.P in Kasegoan, 2012.
10) **7th** Inter-university Conference and Research competition at Dapoli, 2012.
11) Conference on Emerging technology for sustainable developments at Shivaji University, Kolhapur 2012.
12) **1st National level** conference at Vadgaon college of Pharmacy, on Formulation & standardization of Herbal Drugs, 2012.
13) **1st National level** conference at T.K.C.P, Warananagar on Evolving Pharmaceuticals regulatory & quality system framework, 2012.
14) One day research conference and competition DDDT-2012, Bharati Vidyapeeth College of Pharamacy, Kolhapur, 2012.
15) **4th National level** conference **of FDDPR** at Bharati Vidyapeeth College of pharmacy, Kolhapur 2013.
16) Seminar on "Applied Microbiology for Globe protection", UGC sponsored Y.C College, Satara 2013.
17) National Conference on "Opportunities in Pharmaceutical Industries" PHARMEET Mitcon Institute of Management, Pune, 2013.
18) State level conference and competition at Yashoda Technical Campus, Satara, 2013.

19) Universities – Industries Summit at Aurangabad University, related to all age group Avishkar winner and competition, 2013.
20) Conference at Rajarambapu college of Pharmacy ICMR Sponsored, on Model presentation, Kasegaon, 2013.
21) Research competition at Rajasthani Seva Mandal : Abhinav foundation, Mumbai , 2013
22) Student convection "From Campus to Pharma Corporate" AICTE sponsored, Warananagar. 2013.
23) Conference "Analytic technique development" at Pune Bharati Vidyapeeth College of pharmacy, Pune 2013.
24) Conference on H1N1 Vaccine generation at Rajaram College, Kolhapur 2013.
25) Conference on "Anti-cancer and nanotech in healing" at Nipani, Karnataka, 2013.
26) Homi Baba Centre for Science Education, Tata Institute for Fundamental Research, Workshop on Science and Innovation activity Centre, Mumbai, 2013.
27) Workshop on Recent development in Microwave assisted synthesis and rheological studies, TKCP, Warananagar, 2013.
28) National level Meet of IPA On summit of passing of bill for the Kolhapur division at Delhi 2013.
29) PM fellowship workshop, D Y Patil education society, 2016.
30) National conference on Material science, ETNA, D Y Patil education society, 2016.
31) New emerging trend in water resources, (NETWAR), Solapur 2018.

PROFESSIONAL MEMBERSHIPS:

1) Life member of **Indian Pharmaceutical Association (IPA)**.
2) Life member of "**Atmatara foundation**" Kolhapur association for public development and working as The "Vice-president and Pharma/Research Director for the same. www.atmatarafoundation.com.
3) Life membership of "**Maharashtra Industrial Placement Association**" Kolhapur.
4) Life membership of **Bharati Vidyapeeth college of Pharmacy**, Kolhapur, Alumni Association.
5) **Director** of Indian chapter of United Paranormal International India. http://unitedparanormalinternational.ning.com/.

SKILLS:-

- Ability to work under responsibility
- Good analytical skill
- Hard working

- Believe in team work and good leadership qualities
- Good communication skills
- Creative mind
- Designer of layout, poster and PowerPoint presentation
- Using Coral draw 11, 12, 4X

TRAINING UNDER B. PHARM CURRICULUM AND M. PHARM THESIS

- Quality assurance and quality control in Hubli at Hicure Ltd (Parental), July 2010.
- M. Pharm. Thesis title "Probiotics a New generation Dosage form as Nutraceutical and Therapeutic Mediator."

COMPUTER LITERACY:

Designing in Corel draw and Adobe Photoshop, MS Office, MS PowerPoint, MS Excel, Internet, Web Design, You-tube animation etc.

Declaration

I hereby declare that the above written particulars are true to the best of my knowledge and belief.

Place: Kolhapur **Abhinandan Ravsaheb Patil**